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(54) Multiplex ligation dependent amplification assay

(57) Described is a method for detecting the presence of at least one single stranded target nucleic acid in a sample comprising providing said sample or sub-sample thereof, with a first nucleic acid probe complementary to a distinct part of said target nucleic acid and a second nucleic acid probe complementary to a second part of said target nucleic acid located essentially adjacent to said distinct part of said target nucleic acid, wherein said probes further comprise a tag which is essentially non-complementary to said target nucleic acid,

the method further comprising incubating said sample allowing hybridisation of complementary nucleic acids, connecting any essentially adjacent probes, amplifying any connected probe nucleic acid, wherein amplification is initiated by binding of a nucleic acid primer specific for said tag and detecting an amplicon, wherein at least one nucleic acid probe comprises enzymatic template directed polymerised nucleic acid prior to said connecting. Further, nucleic acid probes for use in and a kit for performing the said method are disclosed.

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Description

[0001] The invention relates to the field of biotechnology. In particular, the invention relates to means and methods for the detection and relative quantification of specific nucleic acid sequences in a sample.

5 [0002] There are many methods for the detection of specific nucleic acids in a sample. Most of these methods rely on subjecting sample nucleic acid to a nucleic acid amplification reaction allowing the amplification of the nucleic acid (s) to be detected.

10 [0003] Although many variations exist, most of the methods rely on a nucleic acid amplification reaction. Nucleic acid oligomers are provided to the sample to enable priming of nucleic acid synthesis on specific sites on the nucleic acid. Subsequently nucleic acid is amplified through successive denaturation, hybridisation and nucleic acid polymerisation steps. The sequence of the primers determines where and whether hybridisation with template nucleic acid is possible and thereby whether nucleic acid is amplified.

15 [0004] Detection of an amplified nucleic acid, a so-called amplicon, can occur in many different ways. Non-limiting examples are size fractionation on a gel followed by visualisation of nucleic acid. Alternatively, specific amplified sequence can be detected using a probe specific for a part of the amplified sequence. Combinations of these detection systems are also possible.

20 [0005] Detection of specific nucleic acids in a sample has found many applications. One of these applications is the detection of single nucleotide substitutions in genes. Single nucleotide substitutions are the cause of a significant number of inherited diseases and/or may confer a greater susceptibility to display a certain phenotype such as a disease or an infliction.

25 [0006] Analysis of single nucleotide polymorphisms (SNP's) has important implications in medicine, as not only a considerable number of inherited diseases but also acquired diseases such as cancer are known to be correlated with the presence of point mutations, in the latter case the SNP do not have a homogenous distribution throughout cells in the body but rather have a restricted distributed in some cells of the body.

30 [0007] When it is not, or only superficially, known what sequences to look for in a sample, it is common to use a strategy in which a large variety of different sequences can be detected. This so-called multiplex amplification has various uses. Most commonly such multiplex amplification is used for diagnostic purposes. A problem encountered with the current applications for multiplex amplification is the discrimination of the various amplicons that can result from such an amplification. This is particularly so when the relative abundance of various target nucleic acid in the original sample needs to be determined. In this case it is important that the difference in the number of amplified molecules per amplicon is correlated to the difference in the number of target sequences per amplicon in the sample.

35 [0008] To ensure this correlation, a bias in the amplification of sequences not due to a difference in the relative abundance of target nucleic acids in the sample should be avoided as much as possible.

[0009] Multiplex nucleic acid amplification methods can be divided in methods in which one amplification primer pair is used for all fragments to be amplified such as RAPD, AFLP and differential display techniques, and methods using a different amplification primer pair for each fragment to be amplified. The currently available amplification techniques using only one primer pair for all fragments to be amplified are typically used to amplify a random subset of the nucleic acid fragments present in a sample. Multiplex methods for the amplification of specific targets typically use a different primer pair for each target sequence to be amplified.

40 [0010] The difference in annealing efficiency of different primer pairs result in a strong bias in the amplification of the different amplicons thereby strongly reducing the fidelity of a quantitative multiplex assay. Furthermore the presence of a large number of different primers results in a strongly increased risk of primer dimer formation diminishing the possibility of reproducible amplifying small amounts of target nucleic acids.

45 [0011] In one aspect, the present invention provides means and methods for the amplification of multiple probes hybridised to specific target nucleic acids with the use of only one primer pair. A bias in the amplification due to a difference in the sequence of primers used to amplify can thus be completely avoided.

50 [0012] In one aspect, the present invention provides means and methods for copying sequence information of a target nucleic acid into a DNA template. The method comprises hybridising a single stranded DNA probe to a target nucleic acid wherein the probe, upon separation from not hybridised probe, is used as a template for amplification. The method is of course not only suited for the copying of sequence information of RNA into a DNA template. A DNA target can also be copied into a DNA template. This is advantageous when the DNA target, in case of multiplex amplification, needs to be quantified. As mentioned before, a bias due to difference in primer sequences can be avoided by including into the copying action a nucleic acid tag to which primers can be directed. Thus in this aspect the invention provides a method for the analysis of nucleic acid in a sample comprising providing said sample with one or more DNA probes wherein said probes comprise a first nucleic acid tag and a second nucleic acid tag, optionally denaturing nucleic acid in said sample, incubating said sample to allow hybridisation of complementary nucleic acid in said sample, functionally separating hybridised probes from non-hybridised probes, providing said hybridised probes with at least a first primer, complementary to said first tag, and a second oligomer primer, complementary to said second tag, am-

plifying at least part of said DNA probes after hybridisation and analysing the amplicate for the presence of amplified products.

[0013] Preferably, all the probes comprise the same first tag and the same second tag, thereby excluding completely any bias in the amplification of the probes due to sequence differences in the primers. However, it is of course possible to use probes that comprise different first tags and/or different second tags. In this case it is preferred that the primers are matches for similar priming efficiencies. However, some bias can be tolerated for non quantitative applications or when the bias is known, it can be taken into account in a quantitative application.

[0014] Hybridised probes can be separated from non-hybridised probes in a number of different ways. One way is to fix sample nucleic acid to a solid surface and wash away non-hybridised probes. Washing conditions can be chosen such that essentially only hybridised probes remain associated with the solid surface. The hybridised probes can be collected and used as a template for amplification.

[0015] Removal of non-hybridised probes is not always required. In another aspect the invention provides a method for detecting the presence of single stranded target nucleic acid in a sample comprising providing said sample or sub-sample thereof, with a first nucleic acid probe complementary to a distinct part of said target nucleic acid and a second nucleic acid probe complementary to a second part of said target nucleic acid located essentially adjacent to said distinct part of said target nucleic acid, the method further comprising incubating said sample under conditions that allow hybridisation of complementary nucleic acids, connecting any essentially adjacent probes, amplifying any connected probe nucleic acid and detecting an amplicon, wherein at least one nucleic acid probe comprises enzymatic template directed polymerised nucleic acid prior to said connecting. Preferably, said connecting comprises ligation of the essentially adjacent probes.

[0016] In this aspect of the invention, said first and said second probe can only be amplified exponentially when the probes are connected. Since connection can essentially only take place when the probes are adjacent to each other, exponential amplification, and thereby detection of the amplicon is only possible if said first and said second probe where hybridised to the target nucleic acid. Non hybridised probes are not exponentially amplified and are therefore not detected.

[0017] The length of the complementarity region with the target nucleic acid in the probe is preferably long enough to allow annealing at elevated temperatures. The length of the complementarity region is preferably small enough to substantially reduce hybridisation to only partially complementary nucleic acids. Typically the length of the complementarity region is at least 20 nucleotides.

[0018] In a preferred embodiment said first and/or said second probe further comprise a tag which is essentially non-complementary to said target nucleic acid. Such tag can be used for detection of the resulting amplicon. However, preferably the tags are used for the priming of nucleic acid synthesis in the amplification reaction. Thus preferably, said amplification is initiated by binding of a nucleic acid primer specific for said tag. Preferably, said first and said second probe each comprise a different tag. A tag can be of any size, however, typically a tag comprises a nucleic acid with a length of at least 15 nucleotides. A probe comprising a tag therefore typically comprises a length of 35 or more nucleotides. Amplicons of connected first and second probes typically have a length of at least 70 nucleotides. Preferably, the size of the amplicons is sufficiently large to discriminate amplicons from primer dimers that are often formed in amplification reactions in which only very small amounts of starting template is used.

[0019] A problem, particularly encountered in multiplex amplifications, is the discrimination of the different amplicons that can result from the amplification. Discrimination can be achieved in a number of different ways. One way is to design the multiplex amplification such that the size of each amplicon that can occur, is different. Size fractionation on for instance a gel and determination of the size of the detected amplicon then allows discrimination of the various amplicons. Alternatively, amplicons can be discriminated between on the basis of the respective sequences present in the amplicon. For instance through hybridising amplicons to specific probes. However, the latter method has the disadvantage that additional steps need to be included to detect and/or discriminate the amplicons. In the present invention therefore, it is preferred to discriminate between the various amplicons on the basis of size.

[0020] However, the discrimination of amplicons which differ only slightly in size is difficult. On the other hand longer probes, to allow more differences in size of the resulting amplicons, are not very easily synthesised synthetically. Synthetic production of oligonucleotides has many advantages. It is cheap, reliable and the probe can be made essentially pure. Synthetic generation of the probes however, has a disadvantage when longer probes are required. Probes longer than 60 nucleotides typically suffer from less yield and the reliability of the sequence of the probe becomes a problem. A high reliability of the sequence of a probe is particularly important when already one false nucleotide can give false results.

[0021] In the present invention this problem is overcome by utilising at least one probe comprising nucleic acid that is generated through enzymatic template directed polymerisation, prior to said connecting. Enzymatic template directed polymerisation can be achieved for instance in a cell. It is preferably achieved through the action of a DNA polymerase, RNA polymerase and/or a reverse transcriptase. Such enzymatic template directed polymerisation is capable of generating large stretches of nucleic acid with a high fidelity, thereby enabling the generation of a reliable probe, that is

substantially larger than currently reliably possible with the synthetic methods. A probe comprising nucleic acid that is generated through enzymatic template directed polymerisation is in the present invention further referred to as an enzymatic probe.

[0022] Using at least one enzymatic probe it is possible to increase the size differences between the various amplicons.

[0023] Size differences can be generated by increasing the length of the hybridising region of a probe or by introduction of a stuffer region that is not complementary to the target nucleic acid. By varying the size of the stuffer one can easily design probes that comprise the same hybridisation capacity (wherein the length of complementarity region with the target nucleic acid and the CG/AT content are adjusted to each other), while still being able to discriminate the resulting amplicons by size. The stuffer can of course also be used to introduce a tag, for instance for later discrimination on the basis of sequence.

[0024] As mentioned, a preferred embodiment of the invention comprises the use of a method of the invention for multiplex amplification. In this embodiment of the invention the method further comprises detecting the presence of at least a second distinct target nucleic acid. To enable this it is preferred that said sample or sub-sample thereof, is preferably provided with at least a third nucleic acid probe complementary to a distinct part of said second target nucleic acid. In this case at least two different amplicons can be detected. For instance when said first or said second nucleic acid probe is capable of hybridising to target nucleic acid essentially adjacent to the target nucleic acid of said third nucleic acid probe. Successful connecting of probes can then result in an amplicon resulting from the connection of said first and said second probe and an amplicon resulting from the connection of said first or said second with said third probe. This embodiment of the invention has applications in the detection of for instance SNPs which are different in only one nucleotide. One can chose for instance a first probe capable of hybridising to a common target nucleic acid sequence adjacent or in close proximity to the site of the SNP and a second and third probe capable of hybridising to the site adjacent to the first probe and differing from each other in the nucleotide at the site of the SNP. In case probe 2 and probe 3 are both present at the same concentration and are both able to hybridise to the target nucleic acid sequence under the incubation conditions used, half of the target nucleic acids will hybridise to a probe 1 and a probe 2 and the other half will hybridise to a probe 1 and a probe 3 molecule. One can then exploit the difference in ligation efficiency between perfectly matched and mismatched probes in order to determine the nucleotide present at the site of the SNP. At a certain target molecule either probe 2 or probe 3 will have a mismatch at the site of the SNP which strongly reduces the enzymatic or chemical ligation efficiency and thereby reduces the formation of the corresponding amplicon in the amplification reaction.

[0025] In practice one often would need to further provide said sample with at least a fourth nucleic acid probe complementary to said target nucleic acid located essentially adjacent to said distinct part of said second target nucleic acid. Thus resulting in a possible detection of an amplicon resulting from the connection of said first and said second probe and an amplicon resulting from said third and fourth probe. For enabling detection of each additional target nucleic acid one can similarly provide one or two additional probes. This has applications for the detection of more than one target nucleic acid which need not be in the same chromosomal region.

[0026] One of the applications of SNP analysis is the typing of mutations in the rapidly mutating RNA viruses. RNA viruses including the class of retroviruses are very prone to mutation. Such mutation may among others lead to evasion of the immune system or the development of a relative resistance to medicinal drugs. Particularly, human immunodeficiency virus (HIV) comprises many SNPs.

[0027] When the target nucleic acid comprises RNA than one way to copy sequence information of the target nucleic acid into a DNA template is by using a reverse transcriptase. This retrovirus derived enzyme is capable of generating a DNA strand using RNA as a template. A drawback of using reverse transcriptase is however, that it is an enzymatic process that is susceptible to secondary structures in the template RNA. Moreover, reverse transcriptase activity is notoriously difficult to standardise when long sequences are copied thereby reducing the reliability of an amplification strategy. In one aspect, the present invention provides alternative means and methods to generate amplicons substantially longer than 80 nucleotides and thus substantially longer than primer dimers that are often side products of amplification reactions while needing only 80 nucleotides or less copy sequence of the RNA target.

[0028] To allow connection of essentially adjacent probes through ligation, one possibility is to generate probes that leave no gap upon hybridisation. However, it is also possible to provide at least one additional single stranded nucleic acid complementary to at least one interadjacent part of said target nucleic acid, whereby hybridisation of said additional nucleic acid to said interadjacent part allows the connecting of two adjacent probes. In this embodiment of the invention a gap upon hybridisation of the probes to the target nucleic acid is filled through the hybridisation of said additional single stranded nucleic acid. Upon connecting and amplification the resulting amplicon will comprise the sequence of said additional single stranded nucleic acid. One may choose to have said interadjacent part to be relatively small thus creating an increased difference in the hybridisation efficiency between said one interadjacent part of said target nucleic acid and a nucleic acid that comprises homology with said one interadjacent part of said target nucleic acid, but comprises a sequence which diverges from in one or more nucleotides. In another embodiment of the invention a gap

between probes on said target nucleic acid is filled through extending a 3' end of a hybridised probe or an additional nucleic acid filling part of an interadjacent part, prior to said connecting. This is advantageous as the adequate lengths needed for true multiplex methods are provided by this enzymatic template directed polymerisation while the probes added to the nucleic acid sample may consist of only relatively short synthetically produced oligonucleotides. Applications for this particular embodiment include the determination of the breakpoint sites in chromosomal translocations.

[0029] In a preferred embodiment said ligation is performed with a thermostable nucleic acid ligase capable of being rapidly inactivated above approximately 90 °C. Preferably, said inactivation is achieved in an approximately 10 minutes incubation at 90 °C in a buffer wherein said ligase is normally active at the optimal temperature. Once probes are connected it is preferred that essentially no connecting activity is present during amplification since this is not required and can only introduce ambiguity in the method. Since amplification steps usually require repeated denaturation of template nucleic acid at temperatures above 90 °C it is preferred to remove the connecting activity through said heat incubation. In order to prevent hybridisation of probes to sequences only partially complementary it is preferred to perform the ligation reaction at temperatures of at least 55 °C. Presently, there are no such activities known in the art. The present invention therefore in one aspect provides a method for ligating at least two nucleic acid to each other comprising incubating a sample comprising said nucleic acids with a thermostable nucleic acid ligation enzyme, i.e. with an activity optimum higher than at least 45 °C, under suitable conditions, wherein said ligation enzyme is capable of being essentially inactivated by incubating said sample for 10 minutes at a temperature of approximately 90 °C

[0030] In one embodiment the invention provides a method according to the invention wherein at least one probe is generated by connecting at least two nucleic acids essentially in parallel with said connecting of essentially adjacent probes. Preferably, at least one of said at least two nucleic acid comprises enzymatic template directed polymerised nucleic acid prior to said connecting. This embodiment can in one aspect be used to add a stuffer to the amplicon, whereas not all of said at least one probe needs to be generated through enzymatic template directed polymerisation prior to said connecting.

[0031] In one aspect the invention further provides a nucleic acid probe for use in a method of the invention.

[0032] In another aspect the invention provides a mixture of nucleic acids comprising two or more probes according to the invention.

[0033] In yet another aspect the invention provides a kit for performing a method of the invention, comprising a nucleic acid probe or a mixture of according to the invention.

[0034] In still another aspect, the invention provides a kit comprising a thermostable ligation enzyme of the invention, optionally further comprising a nucleic acid probe and/or a mixture of probes according to the invention.

[0035] The method described herein is referred to as Multiplex Ligation Dependent Amplification (MLDA).

[0036] Single nucleotide substitutions in genes are the cause of a significant number of inherited diseases or confer a greater susceptibility to these diseases. In agriculture, SNP's are increasingly important as genetic markers in plant breeding experiments.

[0037] Recent examples of important SNP's include variants in the BRCA genes predisposing to breast cancer (Struewing et al (1997) N Engl J Med. 336, 1401-1408), a variant in APOE predisposing to dementia (Martinez et al (1998) Arch Neurol. 55, 810-816) and a variant in prothrombin predisposing to a bleeding disorder (de Stefano et al (1998) Blood 91, 3562-3565).

[0038] Detection of single nucleotide polymorphisms can be accomplished by DNA sequencing of an amplified portion of the genome. Traditional DNA sequencing remains however a labour intensive means for detecting only which nucleotide is present at one specific site. In a minority of cases the two different alleles of the SNP differ in the formation or disappearance of a restriction endonuclease recognition site, simplifying the detection of the alleles present.

[0039] The polymerase chain reaction using a primer ending at the SNP site has been used to determine the allele present, as the efficiency of PCR amplification is greatly reduced when one of the two nucleotides at the 3' end of one of the PCR primers has a mismatch with the template (Chehab, et al. (1987) Nature, 329, 293-294).

[0040] In regard of the large number of potential assays on a nucleic acid sample, multiplex analysis methods are to be preferred as splitting the test sample among multiple reaction tubes with e.g. different PCR primer combinations multiply the assay costs.

[0041] Recently a primer extension assay was used for a 12-fold multiplex SNP analysis (Ross, et al(1998) Nature Biotechnology 16, 1347-1351. Genome fragments containing the SNP site were amplified by PCR. Added primers specific for the sequence immediately before the SNP site were extended with only one nucleotide using a polymerase and dideoxynucleotides. The products formed were characterised with respect to their specific mass using MALDI TOF mass spectrometry. Although product detection can be automated, sample handling is complicated and includes a 12 fold multiplex PCR reaction.

[0042] Other investigators have used enzymes to detect or cleave DNA-DNA or DNA-RNA hetero duplexes between target and probe nucleic acids. RNase A has been used e.g. to cleave the RNA strand of DNA-RNA heteroduplexes, resulting in specific smaller fragments (Myers et al. (1985) Science, 230, 1242-1246). Chang & Lu (U.S. patent application 5.683.877 (1995)) used a mismatch repair enzyme for the detection of a specific nucleotide at a SNP position

even if that allele was present in a 100 fold lower frequency as the normal allele.

[0043] An old method using the stability of a duplex between the nucleic acid of interest and an oligonucleotide complementary to the region of interest has attracted much interest in recent years. Such short duplexes are stable only under well defined conditions of temperature and salt concentration. If the oligonucleotide has one or more mismatches with the nucleic acid to be analysed, the stability of the duplex is greatly reduced. With the recent advance of micro-arrays containing large numbers of oligonucleotides, a nucleic acid sample may be analysed for the stability of thousands of short duplexes simultaneously although at a very high cost. This high cost precludes the use of this method for the detection of a limited number of SNP's in large numbers of samples.

[0044] Landegran et al (Science (1988) 241, 1077-1080) and others have used the enzymatic ligation of two adjacent oligonucleotides hybridised to the nucleic acid of interest as a test for the presence or absence of a mismatch at one of the two ends to be joined by the ligase enzyme.

[0045] Dependent on the temperature, buffer-conditions, ligase-enzyme and oligonucleotides used, the difference in ligation efficiency of oligonucleotides that are perfectly matched to the target nucleic acid and mismatched oligonucleotides can be very large.

[0046] Several articles describe a combination of the amplification power of the polymerase chain reaction and the sensitivity to single nucleotide changes of a ligase assay.

[0047] Khanna et al (Oncogene (1999) 18, 27-38) describe an assay combining a multiplex allele specific PCR followed by a multiplex ligation amplification assay to identify 19 single-base mutations in the human K-ras gene. The company Applied Biosystems markets a diagnostic test for the detection of mutations in the human CFTR gene, involved in Cystic Fibrosis. In this assay a 15-plex PCR is followed by a 60-plex oligonucleotide ligation assay. Using three colour analysis on sequence type acrylamide gels 32 different mutations can be detected.

[0048] It is however generally acknowledged that multiplex PCR with the use of one primer pair for each fragment to be amplified is a very difficult to develop method due to the variation in annealing rates of the various primers and the exponentially increase in number of potentially formed primer dimers when using large numbers of primers. Another drawback of this approach is the poor yield and quality of chemically synthesised oligonucleotides longer than 60 bases, which limits the size range of the ligation products and thus limits the number of different products that can be separated in a single lane of the gel.

[0049] In contrast to these methods in which a ligation-assay is performed on amplification products, Zhang and coworkers (U.S. patent application 5,876,924 and PCT application WO 98/04746), Barany and coworkers (PCT application WO9745559A1) and Hsuih et al (1996 ; J.Clin.Microbiology 34, 501-507) developed a method in which the ligation reaction precedes the amplification reaction. In order to study partially degraded nucleic acid samples that could not be used for PCR or RT-PCR amplification, they developed a ligation dependent PCR assay in which the nucleic acid present in the sample was not amplified but only served as a template for the ligation of two probes that could be amplified by PCR after successful ligation. Although the possibility of a multiplex assay with the use of this method was recognised by the authors, again the length of good quality chemically produced oligonucleotides is a limiting factor for true multiplexing as the minimum length of the oligonucleotides needed for this approach is close to the maximum length of good quality chemically synthesised oligonucleotides. Hsuih et al therefore suggested a multiplex application of their ligation dependent PCR by "sorting out amplified probes by hybridising a signature probe to the sequence embedded in each probe that uniquely identifies its target". Nor Hsuih et al nor Barany et al, nor Zhang et al describe a true multiplex detection system capable of detecting more than two specific nucleic acid sequences in an easy to perform assay.

[0050] A second drawback of the use of relatively short chemically derived oligonucleotides is that the length of some of the specific amplification products will be close to the length of primer dimers that are often generated in PCR amplification reactions especially when starting with low amounts of starting material. Hsuih et al therefore used semi-nested primers for the amplification reaction in order to increase sensitivity. Zhang et al developed amplification techniques relying on circularizable probes and using rolling circle extension of a single primer annealed to the circularised probe. The long products obtained could serve as a template for a subsequent PCR amplification reaction.

[0051] A third important drawback of the approach used by Hsuih et al and Zhang et al is that RNA is a very bad template for the ligation of two DNA oligomers (Engler, M.J. & Richardson, C.C., 1982, In P.D. Boyer (ed), The Enzymes, vol. 15, Academic Press Inc., New York). ATP requiring DNA ligases such as the T4 DNA ligase used by Zhang and coworkers are capable of ligating two DNA oligomers annealed to adjacent sites of an RNA molecule, but the reaction is inefficient and requires special incubation conditions such as replacement of Mg ions by Manganese. NAD requiring DNA ligases such as the Taq DNA-ligase, Tth-DNA ligase, and ligase-65 which is used in the current invention are not capable of ligating two DNA oligonucleotides annealed to adjacent sites of an RNA molecule. These ligases are however preferred for the specific ligation of these molecules as they are active at temperatures of 60-65 °C at which nonspecific annealing / ligation is reduced.

[0052] Methods described in the art are not very suited for detecting which nucleotide is present at the SNP site for a large number of SNP's simultaneously in an easy to perform and robust test with unequivocal results, at low cost,

and requiring only a small amount of nucleic acid sample.

[0053] Accordingly there is a need for an improved method for the simultaneous determination of the nucleotide present at large numbers of SNP sites.

[0054] Some of the embodiments the invention have in common with the methods described by Hsuih et al (1996; J.Clin.Microbiology 34, 501-507) and Zhang et al (US patent 5.876.924 and PCT application WO 98/04746) that a ligation dependent amplification reaction is used to detect specific nucleic acid sequences in a sample. The current invention however solves the above mentioned limitations of the old methods by using long enzymatically produced oligonucleotides as well as chemically produced relatively short oligonucleotides for the ligation reaction and can be used for true multiplex detection of specific nucleic acid sequences in a single "one-tube" assay.

[0055] DNA rearrangements as well as amplification or deletion of large segments of chromosomal regions due to genetic instability are frequently linked to neoplasia. Chromosome deletions are commonly believed to represent the second genetic inactivation step consistent with the two-hit theory of carcinogenesis. These deletions are usually detected by loss of heterozygosity (LOH) of micro-satellite sequences which is a method difficult to perform on a large number of genes, or by cytogenetic analysis for instance by fluorescent in situ hybridisation ("FISH") which requires specialised expertise, is time consuming and requires large probes to analyse for suspected deleted or amplified regions.

[0056] The current invention can be used to detect amplification of chromosomal regions or loss of heterozygosity at 10-100 sites simultaneously. In addition the relative number of copies of particular genes can be determined without knowledge of micro-satellites or SNP's in these genes. Thus the MLDA process of the current invention constitutes a significant advance over prior processes.

[0057] It is an object of the instant invention to provide a sensitive and accurate method for the simultaneous detection and relative quantification of specific nucleic acid sequences present in a nucleic acid sample.

[0058] In one embodiment this was attained through the development of a method comprising :

(a) obtaining single stranded target nucleic acid sequences;

(b) hybridising probe oligonucleotides to the target nucleic acid. For each nucleic acid sequence of interest one oligonucleotide (type A probe) is provided having a specific length, preferably between 45 and 600 nucleotides, and having at one end a sequence that is capable of hybridising to the nucleic acid sequence of interest and containing, at a certain distance from the hybridising sequence, a sequence-tag X which is common to all type A probes.

For each target sequence of interest also a second oligonucleotide (type B probe) is provided. Each type B-probe is also capable of hybridising to the nucleic acid sequence of interest directly adjacent to the type A probe, and each type B probe contains a sequence-tag Y common to all type B probes. The concentration of probes used is approximately 1 femtomol / 10 ul which is 10.000 times lower than primer concentrations in typical PCR reactions.

In case the purpose of the assay is to determine which nucleotide is present at the site of an SNP, for each SNP one type A probe is provided that is capable of hybridising to a sequence directly adjacent to the SNP site of the target sequence. Furthermore at least two type B probes that are capable of hybridising to the sequence adjacent to the site occupied by the type A probe on the target sequence are provided for each SNP. These type B-probes differ from each other in length and in the nucleotide at the SNP site.

For each type B probe the distance between the sequence tags X or Y and the ligation site is carefully chosen as it will be used for identification of the probes.

(c) Ligation of the hybridised oligonucleotides : When to a target both a type A and a type B probe are hybridised they are adjacent and may be ligated to each other chemically or by a ligase enzyme. The efficiency of enzymatic ligation is high when no mismatch is present between the target and the probes at the site of the SNP. When designing probes for a particular target nucleic acid, care is taken to prevent binding of the probes to non-unique nucleic acids such as sequences occurring in pseudogenes, repeated DNA etc.

(d) amplifying the ligated probe oligonucleotides using sequence tags X and Y. In a preferred embodiment of the current invention, the polymerase chain reaction (PCR) is used for the amplification of ligated probes. As non-ligated probes contain only one of the two sequence tags, they cannot be amplified exponentially.

(e) separating the amplified nucleic acid fragments according to length and detecting the amplified nucleic acid fragments.

[0059] For each SNP two or more different bands may be obtained that differ only a few nucleotides in length dependent on the length of the type B probes. For a single copy gene in a diploid organism only one or two different type B-probes will hybridise to each target site without the formation of a mismatch at the SNP site. These oligonucleotides will be ligated with the greatest efficiency and will give rise to one or two stronger bands compared to other type B probes added for that SNP that have a mismatch with the target sequence.

[0060] As separation techniques for nucleic acids are capable of resolving hundreds of different bands of different

length, a large number of different SNP's may be tested simultaneously, provided that each possible ligation product of an type A and a type B probe is an amplification template that gives rise to an amplification product of unique length. In a preferred embodiment of the current invention, one of the probes used contains an oligonucleotide which is produced by restriction endonuclease digestion of plasmid or phage DNA or by enzymatic template directed nucleotide polymerisation and has a length in excess of 60 nucleotides. In a further preferred embodiment this oligonucleotide is produced by restriction endonuclease digestion of single stranded phage DNA that is made partially double stranded by annealing of a short oligonucleotide to the restriction enzyme recognition site.

5 [0061] As has been shown by Vos et al (European patent application no. 534 858 A1 ; Nucleic Acid Research 23, 4407-4414 (1995)) the polymerase chain reaction is capable of simultaneously amplifying a large number of unrelated nucleic acid fragments with equal efficiency provided that these fragments can be amplified with the same set of PCR primers.

10 [0062] The AFLP method developed by Keygene Company is based on the co-amplification of large numbers of restriction fragments carrying the same oligonucleotide sequence tags attached to their ends by a ligation reaction. The AFLP method is known to be able to discriminate between fragments present on only one, or on both chromosomes 15 of the cells studied. The relative amount of each amplification product reflects the amount present in the sample before amplification.

15 [0063] In the current invention not the target nucleic acids present in the sample are amplified as in the AFLP technique, but (ligated) oligonucleotide probes provided to the sample. Target nucleic acid sequences originally found in the sample being analysed are not amplified because such target sequences do not contain amplification primer-specific tags.

20 [0064] Although the preferred embodiment of the invention uses the polymerase chain reaction for amplification of the probes used, other amplification methods for nucleic acids such as the 3SR and NASBA techniques are also compatible with the current invention.

[0065] An outline of the method described in the current invention is shown in Figures 1-3.

25 [0066] The current invention will also find use in the detection and relative quantification of specific nucleic acid sequences such as mRNA species.

[0067] The techniques currently in use for the (relative) quantification of mRNA of known sequence can be divided in amplification and hybridisation methods.

30 [0068] Amplification techniques generally rely on the enzymatic formation of a cDNA copy of the mRNA followed by an amplification method such as PCR. The number of different mRNA-species that can be analysed simultaneously is very limited (usually only one) and quantification is done by following the amplification reaction with expensive apparatus such as the Roche light cycler. Hybridisation methods range from the proven northern blots which require large amounts of RNA and enable the detection of only a very small number of different mRNA's in a single lane, to methods in which large numbers of specific cDNA fragments are immobilised such as the Atlas Human cDNA Expression Array 35 of the Clontech company. In addition some hybridisation devices containing thousands of immobilised oligonucleotides have become available (DNA-chips). None of these hybridisation methods are sensitive enough for the analysis of small samples (less than 0.5 ug total RNA). In addition they are very expensive (atlas arrays, DNA-chips), require expensive apparatus (DNA-chips), require the RNA sample to be radioactive or fluorescent labelled (atlas arrays, DNA-chips) or are time consuming (Northern blots).

40 [0069] A simple method for the relative quantitation of different mRNA's will be useful for many purposes. Different types of cancer may be distinguished more easily. As each type of cancer may respond differently on medication, the precise molecular characterisation of a tumour is extremely important. Tissues used for transplantation are characterised by the presence of a large number of antigens. Detection of the corresponding mRNA may simplify the characterisation of tissues. Apart from the lack of sensitivity and/or the impossibility of multiplex detection, all mentioned 45 techniques have in common that great technical expertise is needed to perform them and evaluate their results. This makes these methods not suited for introduction into hospital laboratories for routine analysis. There is thus a need for a simple, reliable and robust method for the detection and relative quantification of mRNA species.

[0070] The present invention discloses a general method for the detection and relative quantification of nucleic acid species in a wide variety of samples. The methods of the invention do not rely on the synthesis of a long cDNA of the 50 target RNA and are therefore less dependent on the intactness of the RNA sample and are more robust and reliable than the current methods in the art. In addition, the methods described in the current invention are easy to use "one tube" assays suitable for introduction into routine laboratories.

[0071] Another application of the current invention is the detection of pathogens in a sample. There are many different 55 pathogens that can contaminate food samples or be present in clinical samples. Determination of even minor quantities of a pathogen can be accomplished using nucleic acid amplification methods such as PCR, RT-PCR and 3SR. However, for these purposes, considering the wide variety of potential pathogens, a large number of different primer sets need to be used and their performance optimised. Although possible, this is a lengthy process. In addition, very often not all primer sets can be added in one reaction mix thus necessitating different reactions for full coverage of the potential

pathogens. With the present invention it is possible to scrutinise the presence or absence of a large number of different pathogens in a sample. This can be accomplished by analysing RNA or DNA in a sample.

[0072] As much is known of the sequence of the tRNA's and ribosomal RNA's of different species, this information may be used to design oligonucleotides that will be aligned on either (cDNA of) these abundant RNA species, or the DNA coding for them. The resulting ligation finger-print may provide enough information to identify the specific strain or species from which the nucleic acid was derived. Due to the high copy number of tRNA's and ribosomal RNA molecules, sensitivity of detection techniques can be extremely high.

BRIEF DESCRIPTION OF THE DRAWINGS

- 10 [0073] Figures 1, 2 and 3 show graphic outlines of the MLDA invention.
- [0074] Figure 4 shows a graphic outline of a M13 clone used to prepare the long ligation oligonucleotides used in the MLDA procedure.
- 15 [0075] Figure 5 shows a simplified way of performing MLDA with the use of agarose gels for the detection of amplification products.
- [0076] Figures 6 - 11 show the application of the MLDA invention for the detection of mRNA's.
- [0077] Fig. 6: Detection of mRNA's.
- [0078] Fig. 7: Detection of cDNA made with the use of oligo-dT as a reverse transcriptase primer.
- 20 [0079] Fig. 8: Detection of cDNA made with the use of gene specific reverse transcriptase primers.
- [0080] Fig. 9: The use of tagged reverse transcriptase primers.
- [0081] Fig. 10: The use of sequence tagged reverse transcriptase primers.
- [0082] Fig. 11: The use of reverse transcriptase primers that are part of one of the probes.
- [0083] Figure 12 shows the use of the MLDA invention without the use of target specific clones.
- 25 [0084] Figure 13 shows an alternative way of performing the MLDA invention.
- [0085] Figure 14 shows the use of "viagra"-oligonucleotides to reduce internal secondary structures of the probes.
- [0086] Figure 15 shows an outline of the MLDA invention with the use of complete probes made by PCR.
- [0087] Figure 16 and 17 show the results of the MLDA invention for the analysis of the human CFTR gene.
- 30 [0088] Figure 18 shows the results obtained with the MLDA invention for the analysis of total RNA samples from four different human tissues tested for the presence of four different mRNA's.
- [0089] Figure 19 shows the detection of a human mRNA without the use of a specific clone for that mRNA sequence.
- [0090] Figure 20 shows the results obtained with two complete probes on human total RNA samples.
- [0091] Figure 21 and 22 show alternative ways of performing the MLDA invention.
- 35 [0092] Figure 23 shows the use of the MLDA invention for the determination of the breakpoint site in chromosomal rearrangements.
- [0093] Figure 24 shows a list of nucleic acid sequences for nucleic acids referred to.
- [0094] As used herein, the term "DNA polymorphism" refers to the condition in which two or more different nucleotide sequences can exist at a particular site in the DNA.
- [0095] A complementary nucleic acid is capable of hybridising to another nucleic acid under normal hybridisation conditions. It may comprise mismatches at a small minority of the sites.
- 40 [0096] As used herein, "oligonucleotide" indicates any short segment of nucleic acid having a length between 10 up to at least 800 nucleotides. Oligonucleotides can be generated in any matter, including chemical synthesis, restriction endonuclease digestion of plasmids or phage DNA, DNA replication, reverse transcription, or a combination thereof. One or more of the nucleotides can be modified e.g. by addition of a methyl group, a biotin or digoxigenin moiety, a fluorescent tag or by using radioactive nucleotides.
- [0097] As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of nucleic acid sequence synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e. in the presence of different nucleotide triphosphates and a polymerase in an appropriate buffer ("buffer" includes pH, ionic strength, cofactors etc.) and at a suitable temperature. One or more of the nucleotides of the primer can be modified for instance by addition of a methyl group, a biotin or digoxigenin moiety, a fluorescent tag or by using radioactive nucleotides.
- 50 [0098] A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5'end of the primer, with the remainder of the primer sequence being substantially complementary to the strand.
- [0099] As used herein, the term "target sequence" refers to a specific nucleic acid sequence to be detected and / or quantified in the sample to be analysed.
- [0100] As used herein, the term "hot-start" refers to methods used to prevent polymerase activity in amplification reactions until a certain temperature is reached.

- [0101] As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes each of which cut double-stranded DNA at or near a specific nucleotide sequence.
- [0102] As used herein the term "PCR" refers to the polymerase chain reaction (Molis et al U.S.Pat.Nos. 4,683,195, 4,683,202 and 4,800,159). The PCR amplification process results in the exponential increase of discrete DNA fragments whose length is defined by the 5' ends of the oligonucleotide primers.
- [0103] The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "mutant" refers to a gene or gene-product having at one or more sites a different nucleic acid sequence when compared to the wild-type gene or gene product.
- [0104] As used herein, the terms "hybridisation" and "annealing" are used in reference to the pairing of complementary nucleic acids.
- [0105] Conventional techniques of molecular biology and recombinant DNA techniques, which are in the skill of the art, are explained fully in the literature. See, for instance, Sambrook, Fritsch and Maniatis, Molecular Cloning; A Laboratory Manual, Second Edition (1989) and a series, Methods in Enzymology (Academic Press, Inc.).
- [0106] In one aspect the invention is directed towards multiplex detection and relative quantification of target nucleic acid sequences relating to the use of oligonucleotide ligation and co-amplification of 2 - >100 ligation products for the simultaneous identification of the nucleotide present on a large number of specific sites of the target nucleic acid.
- [0107] The instant invention finds utility in the diagnosis of genetic disorders, for instance those that arise from point mutations. The instant invention can also be used for the multiplex detection and relative quantification of specific nucleic acid species such as mRNA's or pathogen nucleic acids.
- [0108] The present invention can be performed on the nucleic acids from all known organisms, and from all nucleic acid containing cells or tissue types of a certain organism. The target nucleic acid can comprise ribo- or desoxyribonucleic acids. Double stranded nucleic acids are rendered single stranded using known techniques. Ribonucleic acids may be partially reverse transcribed.
- [0109] RNA can be used as a target nucleic acid for the alignment of the oligonucleotides to be ligated. For the multiplex detection of the SNP specific nucleotide however it is more convenient to use denatured DNA as a target as the number of targets for each SNP is than almost identical. The amount of a specific mRNA may be higher but is much more variable. If the purpose of the experiment is however the identification of a strain or species, or the detection and relative quantification of specific mRNA's, RNA or preferably cDNA will be a useful target for the alignment of the oligonucleotides to be ligated.
- [0110] RNA can be a ligation template when T4 DNA-ligase is used as the ligation enzyme and preferably in the presence of Mn ions in the buffer ((Hsuih et al (1996) J. Clin.Microbiology).
- [0111] RNA is however a poor template for ligation reactions in which thermophilic NAD⁺ requiring bacterial ligases are used.
- [0112] Our attempts for the detection of the mRNA coding for the human ribosomal protein S24 using probes that could hybridise to directly adjacent sites of the mRNA were not successful (EXAMPLE 1). As only a very small template (40-70 nucleotides) is needed for the ligation reaction of the two probes, RNA can be efficiently reverse transcribed in the region required with the use of a specific primer located very close to the hybridisation sequences of the probes.
- [0113] For the preparation of cDNA from RNA, a reverse transcriptase enzyme, a suitable buffer, a mixture of four dNTP's and a primer can be used. Suitable primers include oligo-dT and a mixture of random oligomers such as commercially available random hexamers. As only cDNA has to be made from the mRNA's of interest, a mixture of primers specific for the genes of interest can be used as e.g. in EXAMPLE 2. These RT primers can also be used for the purification of the RNA sequences of interest from crude homogenates. Suitable "capture" oligonucleotides contain a region complementary to the target RNA at its 3'end, which can be elongated by reverse transcriptase and a tag such as biotin or digoxigenin e.g. at their 5'end. The tags can be used to purify the RNA molecules of interest e.g. with the use of immobilised streptavidine (Hsuih et al; J.of Clin.microbiol. 34, 501-507 (1996)).
- [0114] The primer used for the reverse transcriptase reaction can also be part of one of the probes as shown in Fig. 11. This probe contains a part complementary to a mRNA sequence at its 3'end, which upon annealing can be elongated by reverse transcriptase. After removal of the RNA e.g. by RnaseH, alkali or heat treatment, a hairpin is easily formed between the target specific sequence at the 5'end of the probe and its complementary sequence on the newly formed cDNA. In this case only one more hybridisation event has to take place before an amplifiable oligonucleotide is formed by ligation of the two probes.
- [0115] For multiplex analysis of ligation products using the length of the ligation product to identify the specific ligation products, at least one of the two oligonucleotides will have a length of more than 60 nucleotides in most (but not necessarily all) of the probes. Fragments substantially longer than 60 nucleotides are difficult to synthesise chemically in high yield and high quality. We therefore used fragments derived by restriction endonuclease digestion of plasmids,

phages or phagemids. These fragments typically contain less than one mistake in every 10.000 bp as template directed enzymatic nucleotide polymerisation occurs with high fidelity and is backed in vivo by several repair mechanisms. Alternatively fragments of a sufficient long length and having a sequence tag can be produced by in vitro enzymatic template directed nucleotide polymerisation as described in example 8. The other probe oligonucleotide to be ligated can be smaller and is most easily produced chemically. The SNP is preferably located on the small chemically synthesised fragment as only one phage or plasmid clone has to be produced for each SNP to be tested.

[0116] Chemically synthesised oligonucleotides are made in a 3'- 5' direction. As coupling yield for each nucleotide is usually only 98,5 %, a considerable number of fragments in unpurified oligonucleotides are shorter than the required oligonucleotide. The oligonucleotide end involved in the ligation reaction should however be constant. For the experiment described in example 1 we therefore chose to use chemically synthesised oligo's of which the 3'-end is joined by ligation to the 5'-end of the long (enzymatic produced) fragment. The 5'-end of DNA fragments produced by restriction enzyme digestion is phosphorylated. The smaller chemically synthesised oligonucleotide (type B probe) does not have to be phosphorylated as only the 3'-end is used for the ligation reaction. The SNP site should be close to the end, preferably at the end or at the penultimate site of the chemically synthesised oligonucleotide in order to obtain the largest difference in ligation efficiency between matched and mismatched oligonucleotides.

[0117] In a preferred embodiment, the long enzymatic produced oligonucleotide is made by an amplification reaction such as PCR with the use of two primers, one of which contains a sequence tag at its 5'end. In another preferred embodiment of the invention the long oligonucleotide is produced by restriction enzyme digestion of a plasmid or phage clone. In a further preferred embodiment, the 5'-end of the long fragment (type A probe) to be ligated should be complementary to the target nucleic acid. Some restriction endonucleases, among which the commercially available Bsm 1 isolated from *Bacillus stearothermophilus* NUB36 cleave the DNA outside their DNA recognition site and provide a means to produce oligonucleotides that have a 5' end with perfect complementarity to the target nucleic acid. Other restriction endonucleases such as Sph I and Aat II produce oligonucleotides that have left only one nucleotide of the restriction enzyme recognition site at the 5'end of the fragment produced and can be used for the production of some type A probes.

[0118] The vector for the production of the long ligation fragment can be double stranded, or can be obtained in both single stranded and double stranded form such as M13 phages and phagemids. A double stranded form is required for efficient cloning of the fragments that are complementary to the target nucleic acid sequence. A single stranded form has advantages during the hybridisation procedure as the concentration of the hybridisation probe does not drop during the incubation due to reannealing of the complementary strands. Also the absence of a DNA strand complementary to the probe diminishes the possibility of the formation of primer-dimers during the amplification reaction. Although restriction endonuclease digestion of single stranded DNA is not usually possible, we observed that digestion of single stranded DNA with a short complementary oligonucleotide annealed to the restriction endonuclease site provides a perfect substrate for digestion by Bsm 1 and several other restriction endonucleases. Care has to be observed however in the digestion of these artificial substrates as single stranded DNA is more prone to degradation than double stranded DNA.

[0119] An outline of a clone used for MLDA reactions as in Examples 1-3 is shown in Fig. 4.
[0120] The single Bsm I site of phage M13mp18 is removed. A new Bsm I site is introduced in the region occupied by the multiple cloning site of M13mp18. This Bsm I site and a Sph 1 site can be used to insert an oligonucleotide having sequence complementarity to the target nucleic acid. In a preferred embodiment this target sequence specific oligonucleotide has a length of at least 20 nucleotides and a melting temperature when annealed to its complementary sequence of at least 60 °C. At the 3'end of this fragment a stuffer fragment is inserted such as a fragment of phage Lambda or phage T7 DNA. In a preferred embodiment, the only purpose of this fragment is to obtain a specific length between the Bsm I site and sequence tag X, which is located at the 3'end of this stuffer sequence. In a second preferred embodiment, this stuffer sequence of one or more probes may contain a specific sequence tag used to identify ligation products for instance using real-time quantitative PCR with the use of molecular beacons as marketed by Stratagene Corp., fluorogenic probes such as Taqman probes that are based on the 5'nuclease activity of some heat stable polymerases and are marketed by the PE Biosystems Corporation or fluorescent probes using fluorescence resonance energy transfer (FRET) as used in the lightcyclers of the Roche company. In a third preferred embodiment the stuffer fragment of one or more probes may have a specific melting temperature that may be used to identify amplification products for instance with the use of the light cycler apparatus of the Roche company.

[0121] At the 3'end of sequence tag X an EcoR5 site is located that is used to remove the type A probe from the bulk of the M13mp18 DNA. Each probe used has a different stuffer sequence between the target specific sequence and the sequence tag in order to prevent amplification artefacts. The length between the sequence tag X which is used during the amplification reaction and the Bsm I site combined with the length between sequence tag Y and the 3'end of the other probe determine the length of the amplification product which may be used to identify the amplified probe.

[0122] In a preferred embodiment, the short probes contain a sequence tag Y at their 5'end and a target specific sequence at the 3'end. In a further preferred embodiment this target sequence specific oligonucleotide has a length

between 18 and 45 nucleotides and a melting temperature when annealed to its complementary sequence of at least 55 °C, preferably at least 60 °C. By using for a specific target sequence more than one short probe differing in one, or a few nucleotides close to the 3'end involved in the ligation reaction as well as in the length between sequence tag Y and the 3'end, target nucleic acid sequences at the site of the ligation-reaction can be distinguished as shown in example 1.

[0123] The formation of specific abundant amplification products may be limited by providing for a specific target nucleic acid a (competitor) oligonucleotide capable of annealing to the same target nucleic acid sequence as the probe used. This may be useful when studying target nucleic acids sequences that differ greatly in copy number for instance when using a probe specific for the cDNA copy of an abundant mRNA sequence as well as a probes specific for the cDNA copies of rare mRNA sequences.

[0124] The target nucleic acid is rendered single stranded and exposed to the various added oligonucleotides in order to enable duplex formation. As certain regions of chromosomal DNA may have a very high G/C content, it may be difficult to denature these stretches of DNA in the solutions of high ionic strength that are needed for the annealing of the probes. Addition of salt after the denaturation step is therefore preferred.

[0125] Annealing of the oligonucleotides to the template is faster in buffers of high ionic strength. The salt concentration of the buffer has to be reduced however to approximately 50 - 100 mM after the annealing reaction for optimal ligase activity. Inclusion of certain chemicals such as polyethyleenglycol-6000 may increase both the ligation activity as well as the oligonucleotide hybridisation speed and do not interfere below certain limits with the amplification reaction.

[0126] The duration of the probe annealing is very important. Some probes will hybridise faster than others. This is due to a difference in length of the hybridising sequence ; the presence or absence of regions with a high % G/C (GC-clamps) ; secondary structure of the probes and / or the target sequence etc. In case the purpose of the experiment is the relative quantification of nucleic acid sequences, care has to be taken that either hybridisation of each probe is complete, or that hybridisation of none of the probes is complete. In case the purpose of the experiment is the relative quantification of genomic DNA sequences, it will be advantageous to prolong the duration of the annealing reaction in order to make sure that all target sequences have probes annealed to them. Most target sequences will be present in diploid form in most tissues and will generate signals of almost equal strength. In case the goal is the relative quantification of mRNA's / cDNA sequences, it may be advantageous to use a high probe concentration for those sequences that are expected in low amounts, and a lower concentration of probes for those sequences that are expected to be abundant in order to equalise the signals obtained for the various probes. In many experiments not the absolute signals obtained for each probe are important but the difference between the results obtained with different samples. If one chooses to lower some probe concentrations, the relative signals obtained with different probes will only be constant if hybridisation of none of the probes is complete.

[0127] Annealing of the probes to the target nucleic acid is concentration dependent and is preferably performed in a small volume in the order of 10 ul. and at temperatures of 50-65 °C in order to prevent annealing of probes to aspecific sites. In order to prevent evaporation the use of a thermocycler with heated lid is preferred.

[0128] In a preferred embodiment, the two target specific sequences of the two probes are complementary to adjacent but not overlapping sites of the target nucleic acid.

[0129] In case probes are used that anneal close to each other but not to adjacent sites on the same nucleic acid strand, the probe with the target specific sequence at its 3' end can be elongated by a polymerase in the presence of a suitable buffer and the four dNTP's in order to make ligation of the two probes possible. As an alternative the gap between the probes can be filled by complementary oligonucleotides that can be ligated to the probes.

[0130] When both oligonucleotides to be ligated are annealed to the target nucleic acid, a covalent phosphate link between the two fragments can be formed enzymatically by a ligase.

[0131] DNA ligases are enzymes capable of forming a covalent phosphate link between two oligonucleotides bound at adjacent sites on a complementary strand. These enzymes use NAD or ATP as a cofactor to seal nicks in ds DNA. Alternatively chemical autoligation of modified DNA-ends can be used to ligate two oligonucleotides bound at adjacent sites on a complementary strand (Xu, Y. & Kool, E.T. (1999), Nucleic Acid Res. 27, 875-881).

[0132] Both chemical as well as enzymatic ligation is much more efficient on perfectly matched oligonucleotide-target nucleic acid complexes compared to complexes in which one or both of the oligonucleotides form a mismatch with the target nucleic acid at, or close to the ligation site (Wu, D.Y. & Wallace, R.B. (1989) Gene, 76, 245-254 ; Xu, Y. & Kool, E.T. (1999), Nucleic Acid Res. 27, 875-881). During recent years many attempts have been made to increase the specificity of the ligation reaction as measured by the relative ligation efficiencies of perfectly matched and mismatched oligonucleotides. The use of longer oligonucleotides, higher reaction temperatures and ligases active at these elevated temperatures has considerably increased specificity. For our purpose we would prefer a ligase that remains active at 50 - 65 °C for prolonged times, but which can be easily inactivated at the higher temperatures used during a PCR reaction. The only ligases commercially available at the moment are enzymes that function only at temperatures below 50 °C such as the DNA ligase encoded by E.coli and by phage T4, and thermostable enzymes that have a half-life of more than 30 minutes at temperatures of 90 °C such as the DNA ligase encoded by Thermus aquaticus.

[0133] For our experiments we purified a NAD requiring DNA ligase from a gram positive bacterium present in our laboratory (Strain MRCH 065). This ligase is designated "Ligase 65" and is commercially available from MRC Holland. Ligase-65 is active at 60-65 °C. In contrast to Tth- and Taq DNA ligase however, the activity of ligase-65 is destroyed more than 90 % by incubation in the optimum reaction buffer for 10 minutes at 95 °C.

5 [0134] In another embodiment, a thermostable ligase such as the ligase from *Thermus aquaticus* may be used and the annealing and ligation reactions can be repeated several times by alternate cycles of heat denaturation and probe annealing. This particular embodiment is more time consuming unless higher concentrations of probes are used to increase the speed of the annealing reaction. Higher concentrations of probes increase however the chance on primer-dimer formation during the amplification reaction. The amount of probes used in the preferred embodiment of the MLDA reaction (approximately 1 - 10 femtoMol in a 10 ul ligase reaction) is more than one magnitude lower than the amounts routinely used in the ligase chain reaction.

10 [0135] Following the ligation reaction, the ligation products consisting of a type A probe covalently joined to a type B probe can be amplified with the use of two oligonucleotide primers, dNTP's and a polymerase, one primer being complementary to one of the sequence tags and the other primer corresponding in sequence to the second sequence tag. The preferred method for amplification is PCR. As shown by Vos et al (Nucleic acid Research 23, 4407-4414; 15 1995), conditions can be found in which small and long fragments containing the same sequence tags are amplified with equal efficiency as they are present in the same amplification reaction and use the same primers. The preferred conditions include a sufficiently long elongation time and the presence of a higher concentration of Taq polymerase than in ordinary PCR reactions. Other amplification methods than PCR such as NASBA or 3SR (Guatelli et al., Proc. 20 Natl. Acad. Sci. USA 87:1874-1878, 1990) can also be used in combination with the current invention. The sequence tags used for the PCR reactions can easily be replaced by RNA polymerase binding sites.

25 [0136] The activity of the polymerase used in the polymerase chain reaction can temporarily be inhibited e.g. by chemical modification of the enzyme or by addition of antibodies to the enzyme. As a result the polymerase activity will be apparent only after heating the sample permitting the development of a one tube test in which the ligase is active at moderate temperatures and is inactivated at high temperatures whereas the polymerase is activated only after the heating step. A so-called hotstart for the PCR reaction is advantageous since in case one of the two PCR primers (complementary to the primer sequence of the long enzymatic produced oligonucleotide) anneals to one of the short probes containing the other PCR primer sequence, a primer dimer is formed upon elongation of the primer. One of the disadvantages of the use of (denatured) double stranded long probes is the increased chance of primer dimer formation 30 as also the second amplification primer can form primer dimers upon annealing to and elongation on the unused strand of the long probe.

[0137] The formation of primer dimers can be further inhibited by using a two-step nested primer amplification reaction. The sequence tag on the long probes used in the examples is 36 nucleotides long which is sufficient for the design of two different primers having limited sequence similarity for use in a nested primer amplification reaction.

35 [0138] In general the amplification conditions for PCR can be equal to the conditions used for AFLP reactions (Vos et al, Nucleic acid Research 23, 4407-4414 ; 1995). Reactions usually stop as a cause of all primers being consumed. Additional amplification cycles have therefore no or only limited influence on the results obtained and results obtained are not dependent on the amount of target nucleic acid in the sample.

40 [0139] There is no need to ensure that each amplification cycle has a 100 % efficiency as long as the chance of each fragment being elongated is equal. As only one primer pair is used in AFLP and in MLDA reactions, this appears to be the case. Care has to be taken however that all primers being elongated during a PCR cycle are also completed. Long fragments require a longer elongation time and higher polymerase concentrations for complete elongation as compared to short fragments. Longer fragments also have a higher chance of remaining unfinished due to a non-complementary nucleotide being incorporated. Addition of a small amount of a proof-reading polymerase such as Pfu to the PCR 45 reaction may prevent this.

[0140] Many PCR protocols as for example "touch-down" PCR deliberately sacrifice the efficiency of the first amplification cycles in order to gain specificity and reduce background. In traditional multiplex PCR using multiple primer pairs this is difficult as the various primer pairs will have different annealing rates especially at temperatures close to, or slightly above the Tm of the primers. As only one primer pair is used in AFLP and MLDA reactions, protocols such as "touchdown" PCR can be used.

50 [0141] Several agents known to increase the speed of the annealing reaction have no or only a limited influence on a PCR reaction. Polyethyleenglycol e.g. has only a limited influence on the PCR reaction at concentrations up to 1 %, implicating that concentrations up to 5 % may be present during the annealing reaction as performed in examples 1-3.

55 [0142] In the examples provided, only 10 ul of the 50 ul ligation reactions are used for the amplification reaction. As the buffer composition during the ligation reaction is very similar to a standard PCR buffer, it is also possible to use the complete volume of the ligation reaction and start the amplification reaction by the addition of primers, dNTP's, a small amount of a non-ionic detergent such as 0.01 % triton X-100 and a heat stable polymerase. These items may also be present during the ligation reaction if a polymerase is used that is activated upon heating to 95 °C. The com-

bination of a heat inactivated ligase, active at 55-65 °C and a heat activated DNA polymerase provides a means to stop the ligation and start the amplification reaction by a heating step without opening the tube.

[0143] The presence of other compounds such as betaine, are known to improve some multiplex PCR amplification reactions and do not severely inhibit the ligation reaction.

5 [0144] Amplification of specific ligation products may be limited by providing to the PCR reaction oligonucleotides complementary to the stuffer region of the long probe of that ligation-product, these oligonucleotides being incapable of being elongated, for instance oligonucleotides with a dideoxy residue at their 3'end.

10 [0145] For some experiments it is advantageous to use PCR conditions that promote the amplification of rare templates as compared to the amplification of more abundant templates in order to obtain bands of almost equal intensity for the different nucleic acid target sequences tested. These PCR conditions may include: (1) The use of higher salt concentrations which promote the annealing of complementary strands and reduce the polymerase activity ; (2) High concentrations of primers ; (3) Reduced annealing / extension temperatures during the last PCR-cycles ; (4) Additives to the PCR buffer such as betaine and DMSO.

15 [0146] In a preferred embodiment detection of the amplification products is accomplished after separation of the fragments by gel-electrophoresis. In some cases it may be desirable to digest the amplification products with one or more restriction endonucleases before gel-electrophoresis in order to differentiate between different possible amplification products.

20 [0147] In our examples we have used a FITC labelled fluorescent primer in order to obtain labelled amplification products and have separated the amplification products with an acrylamide based gel electrophoresis system with a one colour fluorescent detection system. Some automatic sequencers rely on the use of four differently fluorescently labelled primers each having a unique colour signature, enabling the analysis of more than one sample in a single lane and the use of internal size standards. It is however also possible to use PCR primers which are radioactively labelled, or that are labelled with other compounds that can be detected with the use of the appropriate colorimetric or chemiluminescent substrates. In a clinical setting and for general use in many clinical testing laboratories, it is preferable 25 that methods not requiring the use of radiolabeled nucleotides be used.

[0148] In another preferred embodiment, mass spectrometry is used to detect and identify the amplification products.

[0149] In a third preferred embodiment, the melting temperature of the amplification products which can be influenced by the choice of the stuffer fragment is used to identify the amplification-products.

30 [0150] In a fourth preferred embodiment, the presence of a sequence tag on the amplification products is used to detect the amplification products and to analyse the results of the experiment. A sequence tag can easily be incorporated in the stuffer region of the probes and can be used to discriminate e.g. probes specific for wild-type sequences and probes specific for mutant sequences. Separation of the fragments by gel electrophoresis is not necessary as the use of fluorogenic probes and the use of the 5'nuclease activity of some polymerases that can be used in the amplification reaction permits real time quantitative detection of the formation of at least two different sequence tags for 35 instance one tag specific for a control wild-type specific probe and the other tag being specific for one or more different mutant sequences.

[0151] The necessary fluorogenic probes are described for instance by Lee et al (Nucleic Acid Research 21: 3761-3766 ; 1993). Detection of fluorescence during the thermal cycling process can be performed for instance with the use of the ABI Prism 7700 sequence detection System of the PE Biosystems Corp. Other real time detection 40 methods that do not rely on the destruction of sequence tag bound oligonucleotides by the 5'nuclease activity of a polymerase but on the increased fluorescence of some fluorogenic probes (molecular beacons) upon binding to the sequence tag can also be used in the present invention as well as detection probes consisting of two entities, each being complementary to sequences present on one or more amplification-products and each containing a fluorescent moiety wherein fluorescent resonance energy transfer (FRET) occurs upon binding of both entities to the target amplification product.

APPLICATION OF A MLDA ASSAY FOR SNP CHARACTERISATION AND MUTATION DETECTION:

50 [0152] In one embodiment the current invention employs a mixture of probes in one reaction, each probe being specific for one nucleic acid sequence. Each type B probe contains an oligonucleotide that has a region complementary to the target DNA sequence e.g. the sequence essentially flanking the SNP, as well as a common sequence-tag that can be used for the amplification reaction. For each SNP two or more oligonucleotides are used that differ in the nucleotide at the position of the SNP and in the number of nucleotides between the sequence tag used for the amplification reaction and the SNP site. In addition another single stranded DNA fragment (type A probe) is provided that 55 has at one end a nucleotide sequence complementary to the nucleotide sequence flanking the SNP, as well as a sequence tag common to all type A probes which is used in the amplification reaction. Alternatively it is possible to use only one type B probe specific for the rare SNP allele.

[0153] When more than one SNP is analysed, the length of the type A and type B probes can be chosen such that

each possible ligation products results in an amplification product that has a unique size.

[0154] After incubation of the probes and the single stranded template to be analysed under conditions promoting hybridisation of the probes to the target nucleic acid, the mixture is treated with a DNA ligase. In case more than one type B type probes is used, one or more of the type B probes will have a mismatch at the position of the SNP, which is at the end, or close to the end of the type B probe. These probes will be ligated at a lower efficiency to the type A probe than the type B probe(s) that has no mismatch at the SNP position. Upon successful ligation of a type A and a type B probe, a DNA fragment is produced that can be amplified e.g. by PCR using a primer specific for the DNA sequence common to all type A probes and a primer specific for the sequence common to all type B probes.

[0155] NAD requiring ligases are very sensitive to the presence of mismatches between the complementary strands that are closer than 9 bp from the site of ligation. The greatest difference between ligation of completely complementary strands and complexes having a mismatch is however obtained when the mismatch is exactly at the site of ligation.

[0156] Type B-probes have a preferred length of 30-60 nucleotides and differ for instance 2 - 4 nucleotides in length dependent on the nucleotide at the SNP position. Type A probes preferably have a length of 45 - 600 nucleotides and differ for instance in length 6 - 15 nucleotides. For each SNP a different set of two or more type B probes and one type A probe is added. Upon multiplex amplification and detection e.g. on sequencing type polyacrylamide gels, or by mass spectroscopy, a banding pattern is obtained in which the length and the relative intensity of the bands obtained depend on the length of the type A and type B probes and the efficiency of ligation of the different type B probes to the type A probes.

[0157] It has been well established that two oligonucleotides annealed to adjacent sequences on a target nucleic acid are efficiently ligated provided that there is no mismatch between the oligonucleotides and the target nucleic acid close to the ligation site. Thus the type B probe having the best complementarity to the target nucleic acid will be ligated more efficiently to the type A probe than the other type B probes.

[0158] Different oligonucleotide sets for different SNP's can be tested simultaneously provided that each possible amplification-product has a unique length. Using sequencing type electrophoresis systems and multicolour fluores-25cently labelled PCR primers, more than 100 SNP's may be analysed in one lane.

APPLICATION OF A MLDA ASSAY FOR DETECTION OF SPECIFIC NUCLEIC ACID SEQUENCES

[0159] In case the purpose of the experiment is only to detect the presence or absence of a specific nucleic acid sequence, only one A and one B type probe specific for that particular nucleic acid sequence and annealing to adjacent sites on that target nucleic acid need to be provided. Again by changing the length of one or both of the probe oligonucleotides, all ligated oligonucleotides can be detected and identified by virtue of the unique length of the amplification products of the ligation products. Samples can therefore be tested simultaneously for the presence of a large number of nucleic acid sequences in one assay.

[0160] In a preferred embodiment of the current invention, denatured chromosomal DNA is used as a target nucleic acid and two probes are used for the detection of unique non variable sequences of this chromosomal DNA as an internal control. For any mutation to be detected a probe is provided that has the sequence unique for that mutation at or close to one of the ends of the probes that are ligated. No probe specific for wild-type sequences needs to be provided except for the two control probes. In case no allele carrying the mutation is present, only the two control probes will be amplified. Only in case a mutation recognised by one of the probes is present in the target nucleic acid, more bands will be generated. As the number of bands obtained is small, the amplification products can be analysed by rapid and cheap methods such as agarose gel electrophoresis. Samples containing a mutation can be analysed further using methods with higher separating power such as acrylamide type sequencing gels or by sequence analysis of the amplification product. An outline of this MLDA variant is shown in Figure 5. This particular embodiment is described in further detail in Example 6.

[0161] In many cases it may be preferred to detect only one or a few wild-type sequences as a control for the presence of sufficient target nucleic acids and the correct performing of the MLDA assay and a large number of mutant target nucleic acid sequences. In a further preferred embodiment the signal obtained from the wild-type specific probes is reduced by the addition of competing oligonucleotides binding to the same wild-type nucleic acid sequences. As a result the relative amount of signal obtained from the mutant specific probes is increased. In general the wild-type sequences are present in both chromosomal copies while the mutant sequences are present on one chromosome only. In case tumour samples are analysed it must be realised that biopsy material from a tumour can have a significant complement of normal cells. MLDA assays as described in Figure 5 and example 6 can be used to detect mutated nucleic acid sequences in a high background of normal DNA provided that the sequence of the mutation is known.

[0162] MLDA assays as described in Figure 5 and example 6 have an advantage over traditional nucleic acid amplification based detection methods as traditional PCR, 3SR and Nasba in that internal controls are provided for each sample confirming that a negative test result was not due to any error made during preparation of the sample or during sample analysis.

[0163] In another preferred embodiment wild-type and mutant specific probes may be distinguished by the presence or absence of a specific sequence tag in the stuffer region of the probe oligonucleotides. This sequence tag can be used for the binding of complementary labelled oligonucleotides that can be used in real time amplification methods. Useful oligonucleotides are for example the so-called "Molecular Beacons" marketed by Stratagene Corporation and 5 T_Aqman probes marketed by PE Biosystems Corp. both containing a reporter fluorescent dye as well as a quencher dye and dual fluorescently labelled hybridisation probes capable of fluorescence energy transfer as marketed by Roche company for use in the lightcycler. Detection of amplification products containing a specific sequence tags is accomplished by detection of increased fluorescence due to binding of the molecular beacon to the sequence tag or by degradation of target bound taqman probes by the 5' nuclease activity of some polymerases such as Taq polymerase.

10 The advantage of the use of these real time fluorescence detection methods is that labour intensive gel-electrophoresis for the separation of wild-type (control) and mutant specific amplification fragments is avoided and that tubes do not have to be opened after the amplification reaction, diminishing the chance on contamination of other samples that have not yet been amplified. A disadvantage is that only a very limited number of different fluorescence signals can be discriminated as compared to the simultaneous discrimination of more than 50 different amplification products by gel-electrophoresis or mass spectrometry.

15 [0164] Finally it is also possible to use only probes specific for certain sequences and detect amplification products by the appearance of long double stranded DNA for instance by measuring the increased fluorescence of some DNA intercalating dyes such as SYBR Green. The long amplification products formed in the process of the current invention can be easily distinguished from primer dimers for instance by the measurement of the melting temperature after or during the amplification procedure.

20 [0165] MLDA assays as outlined in Figure 5 can easily be combined with the MLDA variant described in example 3 and outlined in Figure 12. Using one long oligonucleotide containing a sequence tag and a mixture of target specific short chemically derived oligonucleotides that can be ligated to each other and to the long oligonucleotide, a multiplex test for the detection of specific nucleic acid sequences can be rapidly developed. In a preferred embodiment such 25 test is used for the detection of relatively rare mutations.

APPLICATION OF a MLDA ASSAY FOR THE RELATIVE QUANTIFICATION OF mRNA SEQUENCES

30 [0166] In case the purpose of the experiment is the relative quantification of a number of different nucleic acids, such as different mRNA's, only one type A and one type B probe specific for each particular nucleic acid sequence and annealing to adjacent sites on that target nucleic acid need to be provided. By careful selection of the length of the probe oligonucleotides, all ligated oligonucleotides can be detected and identified by virtue of the unique length of the amplification products of the ligation product. The relative amounts of most (>90 %) of the probes tested remained identical during the polymerase chain reaction using the PCR conditions as described in example 1, provided that each 35 fragment contains a unique sequence flanked by two sequence tags common to all probes that are used for the annealing of the amplification primers. As the relative amounts of different bands remain unchanged during the amplification reaction, the amount of each amplification product therefore reflects the amount of each ligation product. Using conditions that enable only target nucleic acid directed oligonucleotide ligation, the amount of each amplification product therefore reflects the amount of target nucleic acid present in the sample analysed.

40 [0167] RNA can be a ligation template when T4 DNA-ligase is used as the ligation enzyme, preferably in the presence of Mn ions in the buffer ((Hsuih et al (1996) J. Clin. Microbiology).

45 [0168] RNA is however a poor template for ligation reactions in which thermophilic NAD⁺ requiring bacterial ligases are used. As only a very small template (40-70 nucleotides) is needed for the ligation reaction of the two probes, RNA can be efficiently reverse transcribed in the region required with the use of a specific primer located very close to the hybridisation sequences of the probes (Fig. 8). Alternatively the RNA can be reverse transcribed with the use of oligo-dT (Fig. 7) or a mixture of small oligonucleotides of random sequence as a primer. Finally a reverse transcription primer sequence can be part of one of the two probes (Fig. 11). After reverse transcription, the RNA can be removed from the cDNA by RnaseH treatment or by heating. In case a probe is used with a target (cDNA) specific sequence at its 5'-end, a reverse transcription primer sequence at its 3'-end and the oligomer tag used for the amplification reaction in-between, a (large) hairpin will be formed easily when the target specific sequence anneals to its complementary cDNA sequence (Fig. 11). In this case only one hybridisation event needs to take place before the ligation reaction apart from the rapid hairpin formation.

50 [0169] The primers used for the reverse transcription reaction whether gene-specific or oligo-dT can also be used for the purification of the mRNA's from cell homogenates. Using reverse transcriptase primers with a gene specific sequence at the 3'-end and a hapten such as biotin, digoxigenin or a specific DNA sequence at the 5'-end (Fig. 9), mRNA's can be purified from cell homogenates with the use of immobilised hapten binding agents such as Streptavidin or a complementary DNA-sequence.

55 [0170] One preferred embodiment of the current invention makes use of reverse transcription primers containing a

common sequence tag such as a GT repeat as used e.g. in the oligonucleotides SEQ ID NO. 81-84. These sequence tags can be used before or after the reverse transcription-reaction to enrich the nucleic acid sequences of interest for instance by providing a complementary CA repeat containing a hapten such as biotin and immobilised streptavidine for the binding of the biotin moieties (Figure 10). An advantage of this indirect purification system compared to biotinylated reverse transcription primers is the possibility to remove the enriched nucleic acids from the immobilised streptavidin-biotin complex by heating. Alternatively the CA oligonucleotides can be immobilised directly.

[0171] In general relative quantification of target sequences can be accomplished by using a high enough probe concentration and long enough annealing times in order to make sure that 100 % of the target nucleic acids have probes annealed to the target sequences. This is a preferred embodiment of the MLDA method when using chromosomal DNA as a target nucleic acid. Alternatively relative quantification can be accomplished by ensuring that at none of the target sequences annealing of probes is complete. This is a preferred embodiment of the MLDA invention when the target sequences differ substantially in copy number as is the case between different mRNA's.

[0172] The relative amount of specific amplification products A can be reduced compared to the amount of other amplification products by providing a competitor oligonucleotide capable of annealing to a target sequence, preventing the annealing of one or both of the probes specific for that target sequence. By using mixtures of target specific probes and competing oligonucleotides binding to the same target sequence, the relative amount of amplification products from abundant mRNA's can be reduced enabling the use of annealing times that are long enough to allow close to 100 % of the target sequences having probes or competing oligonucleotides annealed to them.

[0173] The application of the MLDA invention for the relative quantification of mRNA's is described in Example 2. An outline of the method is presented in Figures 6-11.

THE USE OF A MLDA ASSAY WITHOUT TARGET SPECIFIC CLONES.

[0174] If the current invention is applied to the analysis of one, or a small number of target nucleic acids, only relatively short (40-80 bp.) chemically produced oligonucleotides are required. For the simultaneous analysis of larger numbers of target nucleic acids however, long (60-600 nucleotides) oligonucleotides are required. With the current technology for the chemical synthesis of these molecules, oligonucleotides longer than approximately 80 nucleotides will preferably be enzymatic produced. Although technically no problem for one of ordinary skill in the art, this is time consuming as a new clone has to be produced for every SNP to be tested. We have therefore devised an alternative way to detect the specific nucleotide present at the site of an SNP that requires only SNP specific short (40-60 nucleotides) chemically produced oligonucleotides. This approach can also be used for the other applications of the current invention such as the detection and relative quantification of nucleic acid species, and is outlined in Fig. 12.

[0175] With this approach two ligation events are necessary to produce an amplification template consisting of two target specific oligonucleotides and a chemical or enzymatic produced oligonucleotide that has no relation at all to the target nucleic acid.

[0176] Two target nucleic acid specific oligonucleotides are aligned by the target nucleic acid and are a substrate for chemical or enzymatic ligation. Oligonucleotide 77 contains a sequence complementary to the target nucleic acid as well as sequence tag to be used in the amplification reaction. For SNP detection, a mixture of two or more oligonucleotides 77 can be used which differ in length as well as in the nucleotide present at the SNP site.

[0177] Oligonucleotide 89 contains a sequence complementary to the target nucleic acid adjacent to the target specific sequence present in oligonucleotide 77, as well as a sequence complementary to oligonucleotide 90. Oligonucleotide 89 should be phosphorylated at its 5'end.

[0178] Oligonucleotide 90 contains a sequence complementary to oligonucleotide 89 as well as a sequence complementary to oligonucleotide M227. The function of oligonucleotide 90 is only to align oligonucleotides 89 and M227 in order to make chemical or enzymatic ligation of these oligonucleotides possible.

[0179] Oligonucleotide M227 contains a sequence complementary to oligonucleotide 90 as well as a sequence tag to be used in the amplification reaction. In case many target nucleic acids are analysed simultaneously, oligonucleotide M227 has to be relatively long for some target nucleic acids and may be preferably enzymatic produced. As this oligonucleotide has no target specific sequences, a standard set of oligonucleotides 90 and M227 can be used for many different target sequences.

[0180] For a true multiplex assay, different probes can be used which result after successful template directed ligation in amplification products having different characteristics such as length, mass, sequence, presence of a sequence tag or melting behaviour.

ALTERNATIVE EMBODIMENTS FOR PERFORMING A MLDA ASSAY:

[0181]

5 a) In the experiment described in example 1 we have used chemically synthesised oligo's of which the 3'end is joined by ligase to the 5'end of the long (enzymatic produced) fragment. It is however also possible to use enzymatic produced long oligonucleotides of which the 3' end is joined e.g. with the use of a DNA-ligase to the 5' end of chemically produced short oligonucleotides. An outline of this MLDA variant is presented in Fig. 13. Again the SNP site should be close to the end, preferably at the end or at the penultimate site of the chemically synthesised oligonucleotide in order to obtain the largest difference in ligation efficiency between matched and mismatched oligonucleotides.

10 The 3' end of the long fragment to be ligated should be complementary to the target nucleic acid. This fragment can be produced by restriction endonuclease digestion of a plasmid or phage clone. Some restriction endonucleases, among 15 which the commercially available Sau 3A-I isolated from *Staphylococcus aureus* and Mbo I isolated from *Moraxella bovis* cleave the DNA outside their DNA recognition site and provide a means to produce fragments that have a 3' end with perfect complementarity to the target nucleic acid. Digestion of single stranded DNA obtained e.g. from phage M13 derivatives can be accomplished by rendering the phage nucleic acid partially double stranded at the restriction site by incubation with a complementary oligonucleotide. In case of digestion with Mbo I, the phage DNA has to be produced in a bacterial strain that does not 20 contain a functional dam methylase, such as the *E.coli* JM110 strain available from Stratagene corp.

25 Many restriction endonucleases such as EcoR I and Hind III produce oligonucleotides that leave only one nucleotide of the restriction enzyme recognition site at the 3'end of the fragment produced and can be used for the production of some probes.

An advantage of this alternative approach is that the long oligonucleotide used can be made partially double 30 stranded by incubation with a complementary oligonucleotide and a DNA polymerase. An oligonucleotide that is partially double stranded may be a more efficient hybridisation probe as a result of reduced internal secondary structure.

Disadvantages of this embodiment of the current invention however are the increased risc on formation of 35 long primer dimers and the need to phosphorylate the short chemically synthesised probe oligonucleotides.

b) In the examples provided only probe sets are used of which the two probes to be ligated each have a part complementary to the target nucleic acid and where these target specific sequences hybridise with sequential and contiguous portions of the target nucleic acid. Alternatively, the two type A and type B probes may hybridise to 40 non contiguous portions of the target nucleic acid. The gap between the two probes can be filled before the ligation reaction by one or more other target specific oligonucleotide as depicted in Figure 21, or by a polymerase filling in the gap as depicted in Figure 22. The polymerase should preferably have no or only a low level of strand displacement activity and 5' nuclease activity. In this last embodiment in which a polymerase is used to fill the gap between the two probes, there is no need to use long enzymatic produced probe oligonucleotides in order to obtain amplicons of sufficient length to perform multiplex analysis and to have a sufficient length to distinguish amplicons 45 from primer dimers. In example 11 this embodiment is used to determine the site of chromosomal breakpoints.

c) In case the target DNA is immobilised before or after hybridisation to probes, the non-bound probes can be easily removed and will not be present during the amplification reaction. Although not necessary for most applications, this will reduce the background in case less than 1000 target molecules are present. Immobilisation can be accomplished for instance by cross-linking denatured target nucleic acid to filters as is often accomplished in dot-blot hybridizations. Alternatively the target DNA can be tagged by modification with biotin or digoxigenin residues by commercially available reagents. Before or after hybridisation tagged target nucleic acid can be separated from non tagged nucleic acid probes by well known procedures involving for instance magnetised micro-particles coated with streptavidin or coated with digoxigenin specific antibodies.

50 [0182] In the approach used in EXAMPLES 1-3, a chemically synthesised oligonucleotide (type B probe) is ligated at its 3'-end to the 5'-end of a long enzymatic produced type A probe. This way the probes can be made partially double-stranded next to the part that hybridises to the target nucleic acid by addition of a complementary oligonucleotide (Fig. 14). This "viagra"-oligonucleotide reduces the internal secondary structure of the probe and results in some cases in a faster hybridisation of the probe to its target sequence.

55 [0183] In a further embodiment "full length probes" may be used, consisting of a single oligonucleotide containing the two different sequence tags and giving rise to an amplification product of a specific size. Amplification reactions such as PCR are capable of detecting less than 100 molecules containing the two sequence tags. For many purposes not the absolute signal strength but the relative strengths of the signals obtained with different probes are interesting.

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Amplification reactions are often allowed to proceed for more than the minimum number of cycles needed in order to obtain signals of comparable intensities for different samples that may contain different amounts of target sequences and are stopped when one of the necessary ingredients, usually the amount of primers, becomes limiting.

- 5 [0184] The large number of amplification cycles increases the danger of minor contaminants being amplified to detectable levels when the amount of amplifiable ligation products is very low. A small amount of a "full length probe" e.g. 100 molecules, may therefore be provided to each sample. Detection of the amplification product of this full length probe is a warning that insufficient target specific ligation products were present at the start of the amplification reaction and that the results obtained should be regarded with caution.
- 10 [0185] Complete probes may also be used as spiked internal controls added after or even before purification of the nucleic acids from a sample in order to check the sample preparation and to estimate the absolute amount of the ligation products specific for the target sequences that are present before the amplification reaction.
- 15 [0186] As an alternative to ligation dependent formation of amplifiable molecules, "full length probes" containing not only the two oligomer tags needed for the amplification reaction at a certain distance from each other but also in-between or next to these oligomer tags a sequence capable of hybridising to a target sequence, can be used for multiplex detection of a large number of different target sequences. As in a MLDA assay, each probe can be distinguished by the unique length or mass of its amplification product. In order to obtain probes of sufficient length for 20 multiplex analysis, probes are preferably derived from plasmid or phage DNA by digestion with restriction-endonucleases. Alternatively probes can be made by PCR using suitable primers as shown in Fig. 15. After hybridisation to the target sequences in a sample to be analysed, hybridised probes have to be separated from non-hybridised probes which can be accomplished e.g. by immobilising the sample nucleic acids (dot-blots), biotinylation of sample nucleic acids and binding of these sample nucleic acids + hybridised full length probes to magnetic particles coated with streptavidin, and various other means.
- 25 [0187] In EXAMPLE 8 the results obtained with the use of two full length probes made by PCR with human DNA as the template are described.
- 30 [0188] The method of the present invention is advantageously practised for any set of target nucleic acids using a kit containing two or more probes that can be amplified with the same amplification primers wherein each probe contains a sequence complementary to one of the target nucleic acids. Such kits may also contain, in packaged combination, one or more of the following: a hybridisation/ligation buffer; a ligase enzyme; amplification primers specific for the sequence tags of the probes; and amplification reagents.
- 35 [0189] It will be evident to one of ordinary skill in the art that the invention described herein can be modified and adapted without departing from the spirit and scope thereof.
- [0190] The artisan will further acknowledge that the Examples recited herein are demonstrative only and are not meant to be limiting.

35 EXAMPLE 1.

DETECTION OF MUTATIONS IN THE HUMAN CFTR GENE.

- 40 [0191] For the preparation of long ligatable single stranded oligonucleotides of different length, we used phage M13mp18 which is available from New England Biolabs. The M13mp18 sequence is available from Genbank, accession number X02513. Double strand DNA of M13mp18 was digested with EcoR1 and Hind 3. The oligonucleotides of SEQ ID NO. 8 and SEQ ID NO. 9 which form together a duplex having ends that can ligate to the EcoRI and Hind III sites of the digested M13mp18 was inserted. After ligation and transformation, plaques containing the inserted oligonucleotide were selected and double stranded DNA was prepared of transformant MRCH001.
- 45 [0192] Double stranded DNA of this virus was digested with Nco I and Acc I. The oligonucleotides of SEQ ID NO. 10 and SEQ ID NO. 11 which form together a duplex having ends that can ligate to Nco I and Acc I sites of the digested MRCH001 was inserted. After ligation and transformation, plaques containing the inserted oligonucleotide were selected and double stranded DNA was prepared of transformant MRCH002. M13mp18 contains a Bsm 1 recognition site at position 1745-1750 which we removed from phage MRCH002 by changing the T-nucleotide at position 1748 into a C-nucleotide.
- 50 [0193] A primer (SEQ ID NO. 12) was annealed to single stranded M13mp18 DNA. This primer was elongated by the Klenow fragment of DNA Polymerase I. After closing the resulting double stranded DNA with T4-DNA ligase, the DNA was heated 5 minutes to 95 °C in the presence of 10 pMol of an oligonucleotide (SEQ ID NO. 13). This oligonucleotide was again elongated by Klenow fragment and the resulting d.s. DNA preparation was transformed in E.coli strain JM109 (Promega). Transformants were cultured together in one bottle for 5 hrs. Double stranded virus DNA was purified from the mixture of transformants and was digested with Bsm I. The digested DNA was again transformed in E.coli strain JM109, and virus plaques were tested for the presence of a Bsm 1 site. One transformant (MRCH106) not containing a Bsm I site was selected.

[0194] Double stranded DNA of this virus was digested with Nco I and EcoR I. The oligonucleotides of SEQ ID NO. 14 and SEQ ID NO. 15 which form together a duplex having ends that can ligate to Nco I and Hind 3 sites of the digested MRCH106 was inserted. After ligation and transformation, plaques containing the inserted oligonucleotide were selected and double stranded DNA was prepared of transformant MRCH107.

5 [0195] Double stranded DNA of MRCH107 was digested with PinA1 and Acc1 and the oligonucleotide SEQ ID NO. 16 + SEQ ID NO. 17 which together form a duplex having ends that can ligate to PinA1 and Acc 1 digested MRCH107 DNA was inserted. After ligation and transformation, plaques containing the inserted oligonucleotide were selected and double stranded DNA was prepared of transformant MRCH214.

10 [0196] The result of these steps is a M13mp18 derivative that lacks the Bsm I site at position 1745-1750 and has the sequence shown in SEQ ID NO. 18 inserted in the EcoR 1 and Hind 3 sites of M13mp18.

15 [0197] Four different PCR fragments derived from phage T7 DNA with the use of the following primer pairs were inserted in MRCH214 : SEQ ID NO. 19 + 20 ; 21 + 22 ; 23 + 24 or 25 + 26. These 4 PCR fragments were digested with Sph 1 and Xba 1 and ligated to Sph 1 and Xba 1 digested d.s.DNA of phage MRCH214. Primers SEQ ID NO. 19, 21, 23 and 25 have an Sph 1 site close to their 5'end. Primers SEQ ID NO. 20, 22, 24 and 26 have an Xba 1 site close to their 5'end. Phage T7 is available from the American Type Culture Collection. The T7 DNA sequence is available from Genbank as Acc. nr. V01146. In addition two oligonucleotides SEQ ID NO. 27 and 28 which together form a duplex having ends that can ligate to Sph 1 and Xba 1 sticky ends was inserted in Sph 1 and Xba 1 digested MRCH214 DNA. As a result five different phages were obtained that each have a DNA sequence of different length between the Bsm 1 site and the nucleotides 77 - 112 (sequence tag Y) of SEQ ID NO. 18.

20 [0198] MRCH228 has a 34 bp T7 fragment inserted ; MRCH266 has a 79 bp T7 fragment inserted ; MRCH273 has a 151 bp T7 fragment inserted ; MRCH285 has a 310 bp T7 fragment inserted and MRCH113 contains a 349 bp T7 fragment.

[0199] The important features of these phages are depicted in Fig. 4 and can be summarised as follows :

25 1) A double stranded DNA fragment A having a CATG overhang at the 3'end of one oligonucleotide and a GG overhang on the other oligonucleotide, and having a sequence complementary to the sequence of interest can be inserted in the phages after digesting the double stranded phage DNA with Bsm 1 and Sph 1.

30 2) When single stranded DNA of the resulting clones is annealed with oligonucleotides of approx. 20 nucleotides that are complementary to the Bsm 1 site of these clones

and the EcoR5 site at position 110-115 of SEQ. ID 18 and their flanking regions, the single stranded DNA can be digested with Bsm 1 and EcoR5 and single stranded fragments are obtained that have the sequences of oligonucleotide A at their 5'-end and a specific length between the 5'-end and the sequence tag Y that is complementary to one of the primers used in the amplification reaction. The oligonucleotide used for digesting the EcoR5 site is shown in SEQ ID NO. 29.

35 3) Each of the 5 phages made produces blue plaques on agar plates containing IPTG and X-gal. Upon insertion of an oligonucleotide X with a length that is not (2 + a multiple of 3), white plaques are obtained.

40 [0200] In each of the five clones containing a T7 stuffer fragment an oligonucleotide was inserted into Bsm 1 and Sph 1 digested double stranded DNA. Each oligonucleotide is identical to the sequence at the 3'-side of a known mutation in the human CFTR gene (Genbank seq. nr. M55108 - M55130).

45 [0201] The partially complementary oligonucleotides SEQ ID NO. 30 and 31 were inserted in the Bsm 1 and Sph 1 sites of MRCH228 ; SEQ ID NO. 32 + 33 in MRCH266 ; SEQ ID NO. 34 + 35 in MRCH273 ; SEQ ID NO. 36 + 37 in MRCH285 and SEQ ID NO. 38 + 39 in MRCH113, resulting respectively in phage clones MRCH231, 236, 243, 258 and 252. Single stranded phage DNA from these five clones was produced as described by Reddy, P. and McKenney K. (Biotechniques 20: 854-860 ; 1996). This single stranded DNA was annealed to two oligonucleotides: Seq ID NO. 29 + 40 for the MRCH231 DNA ; SEQ ID NO. 29 + 41 for MRCH236 DNA ; SEQ ID NO. 29 + 42 for MRCH243 DNA ; SEQ ID NO. 29 + 43 for MRCH258 DNA and SEQ ID NO. 29 + 44 for MRCH252 DNA. Digestion was performed by incubation of 400 pMol of an M13 derivative single stranded DNA with 2.2 nMol of each of the two oligonucleotides and 8000 units EcoR5 in 10 mM Tris-HCl pH 7,6 ; 100 mM KCl ; 10 mM MgCl₂ and 1 mM Dithiothreitol at 37 °C. After incubation for 30 minutes, 4000 units Bsm 1 was added and the temperature raised to 50 °C. After incubation for another 30 minutes the digested DNA was phenol extracted, ethanol precipitated and dissolved in TE.

50 [0202] For each mutation / SNP to be tested, two oligonucleotides were synthesised that have a common part used to amplify ligated oligonucleotides (Sequence tag X), and a part complementary to the CFTR sequence at the position of the mutation.

[0203] These oligonucleotides differ in length (4 bp) and in the nucleotide present at the site of the mutation. The site of the mutation is at the penultimate position or at the 3'end of the oligonucleotide.

[0204] Oligonucleotides SEQ ID NO. 45 and 46 can anneal to a site adjacent to the insert of clone M231 on CFTR

wild-type DNA or to DNA containing mutation E60X of the CFTR gene respectively. Oligonucleotides SEQ ID NO. 47 and 48 can anneal to a site adjacent to the insert of clone M236 on CFTR wild-type DNA or to DNA containing mutation 621+1G>T of the CFTR gene respectively. Oligonucleotides SEQ ID NO. 49 and 50 can anneal to a site adjacent to the insert of clone M243 on CFTR wild-type DNA or to DNA containing mutation deltaF508 of the CFTR gene respectively. Oligonucleotides SEQ ID NO. 51 and 52 can anneal to a site adjacent to the insert of clone M258 on CFTR wild-type DNA or to DNA containing mutation 3659delC of the CFTR gene respectively. Oligonucleotides SEQ ID NO. 53 and 54 can anneal to a site adjacent to the insert of clone M252 on CFTR wild-type DNA or to DNA containing mutation 2184delA of the CFTR gene respectively.

5 [0205] DNA of five different humans (50 ng in TE) was mixed in a 200 ul vial with 4 FemtoMol of each of the five digested phage DNA's and 10 pMol of each of the ten oligonucleotides designated SEQ.ID. 45 - 54 in a final volume of 8.5 ul. DNA was denatured by heating for 5 minutes at 95 °C in a thermocycler with heated lid. To the mixture was added 1.5 ul salt mix : 1500 mM KCl ; 300 mM Tris-HCl pH 8.5 ; 1 mM EDTA. Annealing of the probes to the target DNA was for 6 hrs. at 60 °C in a thermocycler with heated lid. To the mixture was added 40 ul dilution-buffer (2 mM MgCl₂ ; 1 mM NAD+) and 10 units Ligase-65. The mixture was incubated for 15 minutes at 60 °C followed by 5 minutes at 95 °C. 10 ul of the mixture was used as a template for a PCR reaction in a 50 ul volume containing 2 units Taq polymerase ; 15 mM Tris-HCl pH 8.5 ; 50 mM KCl ; 1.5 mM MgCl₂ and 0.01 Triton X-100.

10 [0206] After heating the mixture to 65 °C, 10 pMol of FITC-labelled PCR primer Seq ID NO. 55 ; 10 pMol unlabeled primer SEQ ID NO. 56 and 2.5 nMol of each of the four dNTP's were added.

15 [0207] PCR was performed in 200 ul tubes in a Biometra Uno 2 thermal cycler using
20 the following conditions :

- a) 2.5 minute denaturation at 95 C.
- b) 10 cycles consisting of 30 second denaturation at 95 C ; 30 second annealing at 70 C and 60 second elongation at 72 C.
- 25 c) 40 cycles consisting of 30 second denaturation at 95 C ; 30 second annealing at 60 C and 60 second elongation at 72 C.

[0208] Following the PCR reaction, 2 ul of this reaction was mixed with 2 ul of formamide containing 5 mg/ml blue dextran, heated for 5 minutes at 80 C in order to denature the DNA and was analysed on a 6 % acrylamide gel (acrylamide-bisacrylamide 29 : 1), containing 8 M urea in 100 mM Tris-borate pH 8.3 ; 2 mM EDTA. A Pharmacia ALF apparatus was used to run the gel and detect the fluorescent PCR products. The results obtained are shown in Fig. 16.

[0209] The probes used were designed to give rise upon successful ligation to amplification products of the following lengths when using the above mentioned amplification primers:

- 35 Probes MRCH231 + SEQ ID NO. 45: 148 bp. ;Target: wild-type CFTR gene exon 3.
- Probes MRCH231 + SEQ ID NO. 46: 152 bp. ;Target: CFTR gene mutation E60X
- Probes MRCH236 + SEQ ID NO. 47: 193 bp. ;Target: wild-type CFTR gene intron 4.
- Probes MRCH236 + SEQ ID NO. 48: 197 bp. ;Target CFTR gene, mutation 621+1G>T.
- Probes MRCH243 + SEQ ID NO. 49: 265 bp. ;Target: wild-type CFTR gene exon 10.
- 40 Probes MRCH243 + SEQ ID NO. 50: 269 bp. ;Target: CFTR gene mutation deltaF508.
- Probes MRCH258 + SEQ ID NO. 51: 409 bp. ;Target: wild-type CFTR gene exon 19.
- Probes MRCH258 + SEQ ID NO. 52: 413 bp. ;Target: CFTR gene mutation 3659delC.
- Probes MRCH252 + SEQ ID NO. 53: 454 bp. ;Target: wild-type CFTR gene exon 13.
- Probes MRCH252 + SEQ ID NO. 54: 458 bp. ;Target: CFTR gene mutation 2184delA.

45 [0210] Samples of human chromosomal DNA to be analysed were obtained from the Dept. of Antropogenetica, Free University of Amsterdam, and were known to contain the following mutations in the CFTR gene: Lane 1 of Figure 16: No mutations ; Lane 2: deltaF508 mutation on both chromosomes. Lane 3: deltaF508 mutation on one chromosome only. Lane 4: 3659delC mutation on one chromosome. Lane 5: R117H mutation on one chromosome.

50 [0211] The scale on Figure 16 is not in bp. but in minutes after starting the gel-electrophoresis. As expected, 5 bands were obtained on wild-type DNA corresponding in size with the expected fragment sizes. In lane 2 the third band is running slightly slower through the gel corresponding to a size four bp. longer than the third band of lane 1 and corresponding in size to the fragment expected from the probe specific for the deltaF508 mutation. This same band as well as the wild-type band appear in lane 3, proving that both the wild-type probe as well as the probe specific for the deltaF508 mutation have given rise to an amplification product and thus proving that successful ligation of the probes has occurred. In Lane 4, an extra band has appeared corresponding in size to the probe specific for the 3659delC mutation. As expected no extra bands were observed in Lane 5 as no R117H specific probe was used.

[0212] As the resolving power of acrylamide sequence gels is good enough to use probes that give rise to amplifi-

cation products differing only 4-6 bp in length, the number of probes used in one assay and to be distinguished by the specific length of the amplification products can be 50 or more.

EXAMPLE 2

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a) THE RELATIVE QUANTIFICATION OF mRNA's:

[0213] In order to use the MLDA technique for the detection and relative quantification of mRNA's, probes were made that were complementary to two abundant human mRNA's coding for beta-actin (Genbank acc. nr. M10277) and the S24 ribosomal protein (Genbank acc. nr. U12202).

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[0214] The probes were used in a MLDA assay as described in example 1 using 0.5 ug total human RNA derived from adrenal gland tissue (Clontech) as a ligation template.

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[0215] Attempts were made to use either Ligase-65 at 50 °C or 60 °C or T4-DNA ligase with ATP as a cofactor at 37 °C or 45 °C and with either Mg or Mn as divalent ion during the ligation reaction. None of our attempts was very successful confirming that ligation of two DNA strands annealed to an RNA template is very inefficient when currently known ligases are used. Human chromosomal DNA was a good ligation template for both probes (not shown). Total human RNA gave no signal at all when ligase-65 was used and only a very faint signal when T4-ligase was used. Replacement of Mg by Mn improved the signal somewhat, but detection of the single copy gene sequence in human DNA was much more efficient than the detection of the multiple copy mRNA sequence in human total RNA.

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[0216] As described below much more successful results were obtained by first preparing a cDNA copy of the mRNA's to be detected with the use of reverse transcriptase and a gene specific primer.

b) THE RELATIVE QUANTIFICATION OF cDNA's:

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[0217] Four probes were made that were complementary to cDNA of human mRNA's coding for the S24 ribosomal protein (Genbank acc. nr. U12202), the prostate specific antigen (PSA ; Genbank acc. nr. M27274), thymosin beta-10 (Genbank acc.nr. S54005) and MDA-6 (Gen-bank acc. nr. L25610).

[0218] In the same way as described in Example 1, four different PCR fragments derived from phage T7 DNA with the use of the following primer pairs were inserted in MRCH214 : SEQ ID NO. 57 + 58 ; 59 + 60 ; 61 + 62 or 63 + 64.

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As a result four different M13 derivatives were obtained : MRCH270 has a 115 bp T7 fragment inserted ; MRCH275 has a 169 bp T7 fragment inserted ; MRCH292 has a 208 bp T7 fragment inserted and MRCH202 contains a 304 bp T7 fragment.

[0219] For the S24 probe the partially complementary oligonucleotides SEQ ID NO. 65 + 66 were inserted in vector MRCH202 digested with Bsm I and Sph 1. The resulting clone was designated MRCH213. For the PSA probe the partially complementary oligonucleotides SEQ ID NO. 67 and 68 were inserted in vector MRCH270 digested with Bsm I and Sph 1. The resulting clone was designated MRCH215. For the Thymosin probe the partially complementary oligonucleotides SEQ ID NO. 69 + 70 were inserted in vector MRCH292 digested with Bsm I and Sph 1. The resulting clone was designated MRCH216. For the MDA-6 probe the partially complementary oligonucleotides SEQ ID NO. 71 and 72 were inserted in vector MRCH275 digested with Bsm I and Sph 1. The resulting clone was designated

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MRCH217. Single stranded DNA was prepared from each clone and was digested with Bsm I and EcoRV in the presence of oligonucleotides SEQ ID NO. 29 and 73 (S24) or SEQ ID NO. 29 and 74 (PSA) or SEQ ID NO. 29 and 75 (Thymosin) or SEQ ID NO. 29 and 76 (MDA-6) as described in example 1. These probes were used in a MLDA assay as described in example 1 using oligonucleotide SEQ ID NO. 77 (S24) or SEQ ID NO. 78 (PSA) or SEQ ID NO. 79 (Thymosin) or SEQ ID NO. 80 (MDA-6) as the second probe.

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[0220] A cDNA copy of the RNA to be analysed was made by incubation of 1 ug total RNA from liver, prostate, salivary gland or pancreas tissue (Clontech human total RNA panel V) with one specific primer for each mRNA to be detected. We used primer SEQ ID NO. 81 (S24), SEQ ID NO. 82 (PSA), SEQ ID NO. 83 (Thymosin) and SEQ ID NO. 84 (MDA-6) in order to make a cDNA copy of the specific mRNA's to be analysed, but a mixture of random oligonucleotides or oligo-dT can also be used to prepare a cDNA copy of all mRNA's present in the sample.

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[0221] A mixture of 1 ug RNA and 2.5 pMol of each cDNA primer in a volume of 3.5 ul was incubated for 5 minutes at 70 °C. To this was added 2 ul dNTP mix (2.5 mM of each of the four dNTP's), 1.4 ul concentrated buffer (250 mM Tris-HCl pH 8.3 ; 75 mM KCl ; 15 mM MgCl₂ ; 40 mM Dithiothreitol) and 0.3 ul (60 units) MMLV-Reverse Transcriptase (Promega). Incubation was for 30 minutes at 37 °C in a thermocycler with heated lid followed by denaturation of the cDNA-RNA hybrids by heating 5 minutes at 98 °C. Alternatively a RnaseH treatment can be used to remove the RNA part of the RNA-cDNA hybrid. RnaseH treatment has the advantage that no heat denaturation of the RNA-cDNA hybrid is necessary which is to be preferred in case the RNA preparation to be analysed is contaminated with DNA. Without heat denaturation this DNA will not be accessible for probe annealing and does not need to be removed.

[0222] To the mixture was added 1.4 ul 30x ligase buffer (1500 mM KCl ; 300 mM Tris-HCl pH 8.5 ; 1 mM EDTA)

and 10 femtoMol of each short probe (SEQ ID NO. 77, 78, 79 and 80) and 1 ng digested M13 clones MRCH213 (S24) and MRCH216 (Thymosin) and 10 ng digested M13 clones MRCH215 (PSA) and MRCH217 (MDA6). Final volume was 10 ul. Following incubation at 60 °C for two hrs. in a thermocycler with heated lid in order to accomplish annealing of the probes to the cDNA ligation template, 40 ul dilution-buffer (2 mM MgCl₂; 1 mM NAD+) and 10 units Ligase-65 were added. Ligation was for 15 minutes at 60 °C and was followed by a 5 minutes incubation at 98 °C in order to inactivate the ligase-65. 10 ul of the 50 ul mixture was used as a template for a PCR reaction containing 10 pMol of each PCR primer (Seq ID NO. 55 and 85), 50 uM dNTP's and 2 units Taq polymerase as described in example 1.

[0223] Results are shown in Fig. 18. The probes were designed to produce PCR products of 404 bp (S24), 310 bp (Thymosin), 265 bp (MDA6) and 211 bp (PSA) upon successful template directed ligation of the probes. As expected a strong band of 211 bp corresponding to the PSA probe was detected in samples of prostate RNA. The amounts detected in other tissues was far less. In each sample the amount of amplified thymosin-beta 10 probe was between 64 and 81 % of the amount of Thymosin probe. The MDA6 probe was detected in smaller quantities : between 28 % and 53 % of the amount of S24 probe. Please note that the amount of each probe used was adjusted in order to increase signal from rare mRNA's such as the MDA6 mRNA and to relatively decrease the signal obtained from abundant mRNA's such as the S24 mRNA. Control reactions lacking RNA were blanc.

[0224] The amount of PSA mRNA can be compared to the results of Ishikawa et al (Jap. J. of Clin. Oncology, 28, 723-728 ; 1998). Using quantitative dot blot hybridisation, they found a very high expression in prostate RNA, but also expression of PSA mRNA (although at a 28 fold level) in Salivary gland RNA, Pancreatic RNA (48 x lower level) and in many other tissues. This corresponds well with the results obtained by us using the MLDA method. The PSA signal obtained with prostate total RNA was 115 % of the S24 signal strength. In salivary gland RNA 20 % ; In pancreas RNA 8 % and in liver RNA only 2 %. The signal obtained after PCR amplification with MLDA is non linear with the amount before amplification especially when using a limited number of probes. During the final amplification cycles annealing of complementary probes competes with primer annealing for abundant fragments. This is prevented by using larger number of probes as the amplification reaction stops by depletion of primers before extremely high amounts of fragments are produced.

[0225] The primers used for the reverse transcription reaction whether gene-specific or oligo-dT can also be used for the purification of the mRNA's from cell homogenates. Using primers with a gene specific sequence at the 3'-end and a hapten such as biotin, digoxigenin or a specific DNA sequence at the 5'-end, mRNA's can be purified from cell homogenates with the use of immobilised hapten binding agents such as Streptavidin.

EXAMPLE 3

DETECTION OF mRNA's WITHOUT SEQUENCE SPECIFIC CLONES.

[0226] In order to detect the S24 mRNA without the use of an enzymatic produced oligonucleotide containing a S24 specific DNA sequence, we produced single stranded DNA from M13 clone MRCH227. This clone contains a 268 bp. stuffer fragment derived from phage T7 inserted in M13 derivative MRCH214 described in example 1. Double stranded DNA of MRCH214 as well as a DNA fragment obtained by PCR from a T7 DNA template using primer SEQ ID NO. 86 and 87 were both digested with Xba 1 and Sph 1 and ligated. An M13 clone containing the 268 bp T7 insert was designated MRCH227.

[0227] MRCH227 single stranded DNA was digested with EcoR5 and SpaH1 which is a true isoschizomer of Sph 1. Digestion was performed by incubation of 400 pMol MRCH227 single stranded DNA with 2.2 nMol of each of the oligonucleotides SEQ ID NO. 29 and SEQ ID NO. 88 and 8000 units each of EcoR5 and SpaH1 in 10 mM Tris-HCl pH 7,6 ; 100 mM KCl ; 10 mM MgCl₂ and 1 mM Dithiothreitol at 37 °C. Following digestion for 1 hr, the DNA was phenol extracted, ethanol precipitated and dissolved in TE.

[0228] A cDNA copy of the S24 mRNA was produced as described in example 2, starting with 50 ng total RNA of human adrenal gland tissue (Clontech) and 100 fMol reverse transcription primer SEQ ID NO. 81. The ligation and PCR reaction were performed as described in example 2 except that the probe used consisted of a mixture of 5 fMol each of three chemically produced oligonucleotides SEQ ID NO. 77, 89 and 90 and 10 ng digested MRCH227 DNA. Oligonucleotide SEQ ID NO. 89 was purified by PAGE and was phosphorylated with the use of T4-polynucleotide kinase. As can be seen in Fig. 12, oligonucleotide SEQ ID NO. 90 binds both the MRCH227 DNA as well as oligonucleotide SEQ ID NO. 89 and functions as a ligation-template. Oligonucleotides SEQ ID NO. 77 and 89 can both be bound to, and aligned by DNA or cDNA containing sequences of the S24 gene. In the presence of S24 cDNA and oligonucleotide SEQ ID NO. 90, the oligonucleotides SEQ ID NO. 77 and 89 as well as the SphI-EcoR5 fragment of clone MRCH227 can be ligated to one molecule which can be amplified using primers Seq. ID. 55 and 85 as described in example 1. The resulting amplification product has a length of 394 bp. and was indeed observed when only 50 ng of human total RNA was used as a template for the cDNA reaction (Fig. 19 lane 2).

[0229] As a control, the S24 mRNA was detected in a 5 ng sample of human total adrenal gland RNA using the probe

described in example 2 consisting of Bsm 1 and EcoR5 digested MRCH213 single stranded DNA and oligonucleotide SEQ ID NO. 77. The amplification fragment obtained has a length of 404 bp and is indeed observed in Lane 1.

[0230] The sensitivity of the assay with this latter assay, using two oligonucleotides, appeared to be 8 fold higher than the assay for S24 mRNA using the probe outlined in Fig. 12 that contains 4 oligonucleotides.

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EXAMPLE 4: RELATIVE QUANTIFICATION OF DNA SEQUENCES:

[0231] Using denatured chromosomal DNA from either normal or cancer cells as a ligation template, and probes specific for oncogenes, the relative strength of the signals obtained for each probe after amplification will reflect the relative copy numbers of these oncogenes in the samples used. The absence of an amplification product of a particular probe in the DNA sample derived from cancer cells indicates the loss of both copies of the target sequence. A reduced amount of the amplification product of a particular probe relative to other probes and relative to results obtained with normal cells indicates loss of one copy of the particular target sequence (Loss of heterozygosity). A larger amount of amplification product of a particular probe relative to other probes and relative to results obtained with normal cells indicates amplification of the particular target sequence.

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[0232] The approach is illustrated in lanes 2 and 3 of Figure 17 (Example 6). Two probes recognising wild-type sequences are used as well as one probe recognising the relatively common deltaF508 of the human CFTR gene involved in cystic fibrosis. No amplification product specific for the deltaF508 probe is detectable when analysing DNA from non carriers (Lanes 1, 4 and 5). A small amount of delta F508 amplification product relative to the bands obtained with wild-type specific probes is detected when DNA from a person known to carry this mutation on one chromosome was used (Lane 3). A larger amount of amplification product of the deltaF508 probe as compared to the amplification products of the two wild-type probes, was detected when a sample of DNA from a person homozygote for this mutation was analysed (Lane 2).

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EXAMPLE 5: DETECTION OF GENOMIC IMPRINTING:

[0233] For genes in which the maternal and paternal derived copies differ in one or more single nucleotide polymorphisms, the relative amounts of transcription of these alleles can be determined by using probes specific for these SNP sites and by comparing the relative amounts of the amplification products of each SNP specific probe with the use of either cDNA or denatured chromosomal DNA as a ligation template. For some genes, transcription frequency of the maternal and paternal derived gene copies differ as a result of genomic imprinting.

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EXAMPLE 6: MULTIPLEX DETECTION OF NUCLEIC ACID SEQUENCES:

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[0234] In order to rapidly screen samples for the presence of certain rare mutations / SNP's, probes can be used specific for these mutations / SNP's without the use of probes specific for the wild-type sequence or the common SNP variant. The appearance of an amplification product for these mutation specific probes can e.g. be tested on simple agarose gels or by real time PCR methods.

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[0235] As an example, a series of probes was constructed for different mutations in the human CFTR gene. As in example 1, all probes of enzymatic origin contained non variable sequences adjacent to the site of the mutation, whereas the chemically produced probes contained the site of the mutation at or very close to the 3'end of the oligonucleotide. For each mutation to be detected a enzymatic produced probe 1 was provided (10 ng single stranded M13 clone DNA, digested with Bsm 1 and EcoR5 and each probe containing the same oligonucleotide tag used for the amplification reaction). For each mutation was also provided a chemically produced oligonucleotide probe 2, (4 fMol each) specific for the rare mutation sequence. Each combination of probes 1 and 2 specific for a certain CFTR mutation could give rise upon template directed ligation and subsequent amplification to an amplification product between 300 and 350 bp except for the probes specific for the more common deltaF508 mutation which could give rise to an amplification product of 260 bp.

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[0236] In addition two probes for other wild-type CFTR sequences were provided as a control for correct processing of the samples. These wild-type probes could give rise upon template directed ligation and subsequent amplification to amplification products of respectively 200 and 400 bp. An outline of the assay is provided in Figure 5.

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[0237] Two different PCR fragments derived from phage T7 DNA with the use of the following primer pairs were inserted in the MRCH214 vector described in example 1 : SEQ ID NO. 91 + 92 ; 93 + 94. These 2 PCR fragments were digested with Sph 1 and Xba 1 and ligated to Sph 1 and Xba 1 digested d.s.DNA of phage MRCH214. Primers SEQ ID NO. 91 and 93 have an Sph 1 site close to their 5'end. Primers SEQ ID NO. 92 and 94 have an Xba 1 site close to their 5'end. Phage T7 is available from the American Type Culture Collection. As a result two different phages were obtained that each have a DNA sequence of different length between the Bsm 1 site and the nucleotides 77 - 112 (sequence tag Y) of SEQ ID NO. 18 : MRCH287 has a 331 bp T7 fragment inserted and MRCH294 contains a 232 bp

stuffer T7 fragment.

[0238] In each of the three different clones containing a T7 stuffer fragment an oligonucleotide was inserted into Bsm 1 and Sph 1 digested double stranded DNA. Each oligonucleotide is identical to the sequence at the 3'-side of a known mutation in the human CFTR gene (Genbank seq. nr. M55108 - M55130).

5 [0239] The partially complementary oligonucleotides SEQ ID NO. 95 and 96 were inserted in the Bsm 1 and Sph 1 digested MRCH287 ; SEQ ID NO. 97 + 98 in MRCH292 described in example 2 ; SEQ ID NO. 99 + 100 in MRCH294, resulting respectively in phage clones MRCH261, 308 and 314. Single stranded phage DNA from these three clones was produced as described by Reddy, P. and McKenney K. (Biotechniques 20: 854-860 ; 1996). This single stranded DNA was annealed to two oligonucleotides: Seq ID NO. 29 + 101 for the MRCH261 DNA ; SEQ ID NO. 29 + 102 for MRCH308 DNA and SEQ ID NO. 29 + 103 for MRCH314 DNA. Digestion was performed as described in example 1.

10 [0240] For each mutation to be tested one oligonucleotides was synthesised containing a common part used to amplify ligated oligonucleotides (Sequence tag X), and a part complementary to the CFTR sequence at the position of the mutation. The site of the mutation is at the penultimate position or at the 3'end of the oligonucleotide.

15 [0241] Oligonucleotide SEQ ID NO. 104 can anneal to a site adjacent to the insert of clone M308 on human CFTR gene DNA containing mutation 1717-1G>A. Oligonucleotide SEQ ID NO. 105 can anneal to a site adjacent to the insert of clone M314 on human CFTR gene DNA containing mutation R1162X. Other clones and oligonucleotides used are described in example 1.

20 [0242] DNA of five different humans (50-200 ng in TE) was mixed in a 200 ul vial with the following probes : 4 femtoMol EcoR5 and Bsm I digested MRCH236 DNA described in example 1 + 4 femtoMol oligonucleotide SEQ ID NO. 47 + 6 femtoMol oligonucleotide SEQ ID NO. 48 which together give rise to an amplification product of 193 bp on human DNA having a wild-type sequence in intron 4 of the CFTR gene.

25 [0243] 4 FemtoMol EcoR5 and Bsm I digested MRCH261 DNA + 5 FemtoMol oligonucleotide SEQ ID NO. 106 + 5 FemtoMol oligonucleotide SEQ ID NO. 107 which together give rise to an amplification-product of 436 bp on human DNA having a wild-type sequence in exon 20 of the CFTR gene.

30 [0244] 4 FemtoMol EcoR5 and Bsm I digested MRCH243 DNA described in example 1 +10 FemtoMol oligonucleotide SEQ ID NO. 50 which together give rise to an amplification product of 269 bp on human DNA having a delta F508 mutation (deletion) in exon 10 of the CFTR gene.

[0245] 4 FemtoMol EcoR5 and Bsm I digested MRCH308 DNA + 10 FemtoMol oligonucleotide SEQ ID NO. 104 which together give rise to an amplification product of 326 bp on human DNA having the 1717-1G>A mutation in exon 10 of the CFTR gene.

[0246] 4 FemtoMol EcoR5 and Bsm I digested MRCH314 DNA + 10 FemtoMol oligonucleotide SEQ ID NO. 105 which together give rise to an amplification product of 341 bp on human DNA having the R1162X mutation in exon 19 of the CFTR gene.

35 [0247] Target DNA denaturation, probe annealing, template directed probe ligation and amplification were as described in EXAMPLE 1 except that the annealing reaction was for 16 hrs at 60 °C. Detection of amplification products was performed on ethidium bromide stained 1.8 % agarose gels or on acrylamide gels with fluorescent detection as described in example 1.

40 [0248] Results obtained using 50-200 ng samples of human chromosomal DNA are shown in Fig. 17. In a control sample of human DNA (Lane 1) only the two probes specific for wild-type sequences give rise to an amplification product (193 & 436 bp.). Samples 2 and 3, known to be derived from a deltaF508 homozygote and a deltaF508 heterozygote respectively give in addition to the 193 and 436 bp bands rise to an amplification product of 269 bp. Sample 4, known to be derived from an individual with a 1717-1G>A mutation on one of the chromosomes gives rise to a band of 326 bp in addition to the 193 and 436 bp bands. Sample 5 was derived from an individual having a R1162X allele and gives an amplification product of 341 bp in addition to the 193 and 436 control bands.

45 [0249] The relative amount of amplification product specific for the control sequences has been reduced compared to the amount of amplification product specific for the CFTR mutations by providing an oligonucleotide capable of annealing to the control sequence and preventing the annealing of the control wild-type specific short probe.

EXAMPLE 7:

50 DETECTION OF MICROORGANISMS, PARASITES OR PATHOGENS.

[0250] In order to detect a specific micro-organism, parasite and/or pathogen, probes were designed for a ribosomal RNA sequence that is unique to this organism or a specific variant of this organism. From a sample total nucleic acids are isolated. from this nucleic acid sample, cDNA is prepared using a primer specific for the ribosomal RNA to be detected, reverse transcriptase, dNTP's and a suitable buffer. The cDNA is made single stranded e.g. by RnaseH treatment, alkali treatment or heat denaturation, and used as a ligation template for the probes.

[0251] When using several probes, each specific for a particular organism, or variant of an organism, and each giving

rise to an amplification product of unique size, several nucleic acid sequences / organisms can be identified in a single assay.

[0252] As a control for the sensitivity of the assay and the release of RNA from bacteria during the RNA purification, a specific amount of a unique RNA sequence or a small number of bacteria containing a unique RNA sequence can be added to the sample when starting the nucleic acid purification.

[0253] A microbial cell contains approx. 25.000 ribosomes. When the goal is to detect a minimum of 1 Agrobacterium cell in 10 mg plant tissue, 10.000 - 20.000 copies of a control RNA sequence generated e.g. *in vitro* by methods known in the art, can be added to the 10 mg plant tissue immediately preceding the isolation of the total nucleic acids. To a sample of approx. 1 ug of the purified RNA, reverse transcriptase primers specific for the agrobacterium ribosomal RNA and the control RNA sequences are provided and cDNA is made as described in example 3. Following the reverse transcription reaction a MLDA assay is performed as described in examples 1-3, using e.g. two probes for the control RNA and four probes for regions of the agrobacterium ribosomal RNA that are quite specific for this organism. In the absence of Agrobacterium cells only the probes specific for the control RNA will be amplified and will generate two bands detectable on agarose gels. In the presence of Agrobacterium cells the probes specific for the agrobacterium ribosomal RNA will generate stronger bands than the control bands even when only one bacterial cell was present in the sample. Care has to be taken during nucleic acid isolation that not only the plant cells, but also bacterial cells are disrupted. As an alternative control a defined number of intact microbial cells containing a unique RNA or DNA sequence can be added to the plant sample to be analysed.

20 EXAMPLE 8

COMPLETE PROBES.

[0254] The presence of two different human mRNA in samples of total RNA from 2 different human tissues was determined with the use of two complete probes.

[0255] Samples of total RNA from human prostate and salivary gland were purchased from Clontech Company.

[0256] DNA fragments complementary to the human ribosomal protein S24 and the human prostate specific antigen were made with the use of the polymerase chain reaction using 5 ng human genomic DNA (Promega) as a template and oligonucleotides SEQ ID NO. 1 & 2 (prostate specific antigen) or SEQ ID NO 3 & 4 (ribosomal protein S24) as PCR primers. These primers contain a part complementary to the DNA fragment to be amplified, as well as a part to be used in the detection reaction. PCR conditions were : 2 minutes denaturation at 95 °C ; 30 cycles of 30 seconds denaturation at 95 °C, 30 seconds annealing at 60 °C and 60 seconds elongation at 72 °C. The concentration of the PCR fragments were estimated by ethidium bromide stained agarose gel-electrophoresis with standards. The PCR fragments were used without purification.

[0257] The hybridisation selection reaction was carried out in a 500 ul eppendorf tube containing the following : 12,9 ul deionised formamide, 0.5 ul of the RNA sample (0.5 ug RNA), 0.2 ul each of the two PCR fragments (~5 ng DNA), 0.5 ul biotin-dT43 (SEQ ID NO 7, 50 pMol/ml, dissolved in TE), 1 ul of Rsa 1 digested Lambda DNA (0.5 ug/ml). This mixture was incubated for 5 minutes at 65 °C in order to denature the probe DNA fragments. To this mixture was added 2,6 ul water and 8,1 ul 20 x SSC. Hybridisation was performed by incubation for 2 hrs at 42 °C, after which 80 ul of paramagnetic streptavidin particles were added. The paramagnetic streptavidin particles (SA-PMP) preparation consisted of 40 ul SA-PMP's (Promega) + 40 ul denatured herringsperm DNA (0.5 mg/ml) mixed 20 minutes before use. After incubation at room temperature for 15 minutes in order to allow the binding of the oligo-dT-biotin to the streptavidin-particles, the streptavidin-particles were collected with the use of a magnet (Promega) and washed 4 times at room temperature with 1 ml. 0.5 x SSC + 0.1 % SDS, twice with 0.5 x SSC and twice with 20 x SSC. The particles were transferred to a clean tube and washed twice in 1 x PCR buffer (10 mM Tris-HCl pH 8.5 ; 50 mM KCl and 1.5 mM MgCl₂). The particles were finally suspended in 50 ul 1 x PCR buffer containing 10 ug/ml RNaseA (Roche biochemicals). After incubation for 10 minutes at room temperature, the particles were removed by centrifugation and the supernatant collected. To 10 ul of this supernatant was added 40 ul PCR buffer, 15 pMol of the two PCR primers (SEQ ID NO 5 and 6), 1 unit taq polymerase and dNTP's to a final concentration of 100 uM each. One of the PCR primers (SEQ ID NO 5) is fluorescent labelled as it contains a FITC group covalently bound to its 5'- end.

[0258] After addition of 3 drops paraffin oil, the samples were submitted to 30 cycles of PCR. PCR conditions were : 2.5 minutes 95 °C, followed by 30 cycles of 30 seconds denaturation at 95 °C, 30 seconds annealing at 60 °C and 60 seconds elongation at 95 °C. After mixing with a formamide containing loading solution and denaturation, 0.5 ul of this PCR reaction was analysed on a 6 % acrylamide gel containing 7 M urea in Pharmacia ALF apparatus. The results are shown in FIG. 20. A graphic outline of the method used to detect and quantify mRNA's with the use of complete probes is shown in Figure 15.

[0259] Lanes 1-3 are control reactions. Lane 4 shows the results obtained with RNA from salivary glands. Lane 5 shows the results obtained with RNA from prostate tissue.

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[0260] No RNA was added to the hybridisation reaction for the sample shown in Lane 1. Lane 2 was a reaction with prostate RNA, but no biotin-dT43 was added. In lane 3, both DNA fragments used as probe (Ipgram) were added to the PCR reaction as a positive control.

5 [0261] The two peaks at 218 and 286 minutes correspond to fragment-lengths of 196 and 267 nucleotides as compared to DNA markers in a different lane. This is very close to the expected size of the fragment specific for the prostate specific antigen (195 nucleotides) and the expected size of the ribosomal protein S24 fragment (265 nucleotides). The size of the S24 peak in lane 4 is 12,1 times larger as the size of the psa specific fragment. In lane 5, the size of the S24 peak is only 2,1 times larger than the psa peak. The S24 mRNA is expected to be present in most if not all human tissues. The prostate specific antigen has been detected in several human tissues, but is expected in a relatively larger amount in human prostate tissue.

10 [0262] Identical results were obtained if 100 times less template was used in the amplification reaction, suggesting that even without optimisation of the hybridisation conditions, RNA samples of less than 10 ng might be sufficient for MLDA experiments using complete probes. The control reactions shown in lanes 1 and 2 remained blank when the PCR reaction was extended to 45 cycli.

15 EXAMPLE 9: DETECTION OF DNA METHYLATION:

20 [0263] The genomic DNA of some genes may be more or less modified by cytosine methylation in different tissues or under different growth conditions. Methylation can be detected by digestion with pairs of restriction endonucleases wherein one isoschizomer is sensitive and the other is insensitive to methylation. An example are the enzymes Hpa II and Msp I, both recognising the DNA sequence CCGG and both commercially available from several sources including New England Biolabs. Digestion by Hpa II is blocked when one of the two cytosine residues in the recognition site is methylated, whereas methylation of the internal cytosine residue does not affect Msp I digestion.

25 [0264] By choosing a MLDA probe hybridising to a DNA sequence containing a Hpa II / Msp I site close to the site of ligation, formation of amplifiable ligated probes is prevented when the target DNA is unmethylated and the target DNA is digested with either Hpa II or Msp I before denaturation and hybridisation to the probes, whereas amplifiable ligated probes are obtained when using Hpa II digested target DNA in case part or all of the target DNA is methylated at the internal C of the CCGG sequences.

30 [0265] In case the sample DNA is purified as a chromosomal complex and is not digested with a restriction-endonuclease but with increasing amounts of DNase I, the amount of signal obtained with the various probes will reflect the DNase I hypersensitivity of the particular genes which differs between active and inactive genes and in some cases differ between maternal and paternal inherited copies of a gene.

35 EXAMPLE 10: DETERMINATION OF HAPLOTYPES:

40 [0266] Usually only particular combinations of polymorphisms in a certain chromosomal region are present in a population. Such a combination is called a haplotype. It has for instance been shown that in the Dutch population only five out of eight possible combinations of four polymorphisms in the human TNF gene are present (Crusius JBA et al, Eur. Cytok. Network, 1994;2:168.). In order to determine the haplotypes in a particular chromosomal region, DNA is tested for polymorphisms using an assay as described in example 1. In case more than one locus is heterozygote, primers are designed ending at the outermost polymorphism site, one primer being specific for allele A and the other for allele B. These primers are used to amplify either the chromosomal copy of allele A or the chromosomal copy of allele B, for instance by linear amplification using repeated cycles of denaturation, primer annealing and primer-elongation. Following amplification the DNA is again tested for the polymorphisms present as described in example 1.

45 EXAMPLE 11: DETERMINATION OF THE SITE OF A CHROMOSOMAL BREAKPOINT:

50 [0267] Chromosomal instability is encountered in most cancer cells. Rearrangements in which part of one chromosome is linked to a part of another chromosome is usually detected by histological methods. Some rearrangements are very common in particular types of cancer. Often specific chromosomal regions are involved but exact breakpoints in each region differ between different patients. As an example the chromosomal rearrangement called the Philadelphia chromosome is encountered in many cases of leukemia and involves the linking of part of the BCR gene with part of the ABL gene which are located on different chromosomes. The exact breakpoint can be anywhere in the first intron of these genes and may differ as much as 70.000 bp between different cases. Philadelphia chromosomes can be detected both histologically as well as with the use of RT-PCR on RNA from the cancer cells. Knowing the exact chromosomal breakpoint site is very useful. This information can be used to design primers that can be used to detect DNA fragments specific for cancer cells and not present in wild-type cells of the patient by for instance (nested) PCR. This can make detection of a single cancer cells in more than a thousand other cells possible and can be used to follow

the effect of a therapy.

[0268] In order to determine the exact site of the chromosomal breakpoint, a sample containing chromosomal DNA in single stranded form is provided with a large number of chemically synthesised type A probes. These probes each contain at the 3'end a different sequence complementary to a part of one of the chromosomal region involved at distances of approximately 1000 bp and each probe contains the same sequence tag, 5'of the hybridising sequence. In addition a large number of chemically synthesised probes B are provided to the sample each containing at the 5'end a different sequence complementary to the second chromosomal region involved at distances of approximately 1000 bp and each containing a second sequence tag 3'of the hybridising sequence. Following incubation of the chromosomal DNA with the probes under conditions allowing hybridisation of complementary sequences, the 3'ends of the type A probes are elongated by a DNA polymerase such as sequenase (exo- T7 DNA polymerase), the Klenow fragment of E. Coli DNA polymerase I or the Klenow fragment of Taq polymerase. The DNA polymerase used has preferably no or only a limited amount of strand displacement activity. Probes of which the elongated 3'end have become adjacent to the 5'end of a type B probe can be connected by ligation and can be amplified with the use of a primer complementary to the sequence tag of the type B probe and a primer essentially identical to the sequence tag of the type A probe.

[0269] The resulting amplicons are separated on size and analysed in order to determine which probes have become connected and / or analysed by sequence determination in order to find the exact site of the chromosomal breakpoint.

[0270] If the distance between the different probes is approximately 1000 bp, the resulting amplicons will be between 40 and 2000 bp. In contrast to multiplex amplification methods described in the other examples, only one amplicon is expected. As the size of this amplicon is most often larger than 500 bp, it is possible to chose the sequence tags of the type A and the type B probes to be each others complement thereby permitting the use of only a single primer during the amplification reaction. PCR reactions in which only one primer is used are efficient for amplification of longer fragments and have the advantage that amplification of short fragments such as primer dimers is reduced due to the formation of hairpin-structures in the amplicons.

[0271] One of the main differences between the approach used in this example and ordinary multiplex PCR with multiple primers is that the concentration of probes used in MLDA reactions is typically 10.000 fold lower than in ordinary multiplex PCR reducing the chance on artefacts and formation of primer dimers. Only during the final PCR reaction high concentrations of primers are used, but only of one primer pair specific for the sequence tags common to all probes.

[0272] As an example of this approach, we generated two probes specific for sequences of exon 11 (Genbank acc. Nr. M55116) of the human CFTR gene that bind to target sequences which are at a distance of 95 bp from each other and filled the gap with a polymerase (sequenase ; exo- T7 DNA polymerase) followed by a ligation reaction to connect the probes that became adjacent and an amplification reaction. Using 0.5 ug human chromosomal DNA to provide the target CFTR sequences, we indeed observed the expected 383 bp amplification product consisting of 49 bp of CFTR sequence + sequence tag of probe SEQ ID 113, 95 bp CFTR sequence that was filled in by the polymerase, the 42 bp CFTR sequence of probe M245, the 169 bp stuffer sequence of probe M245, 5 bp between the CFTR sequence and the stuffer region of M234 and 23 bp of primer SEQ ID 56. The presence of the CFTR sequence between the probes was confirmed by digestion of the amplification product with Dra III which has a recognition site at nucleotide 350-358 of sequence M55116, producing fragments of 298 and 85 bp. (not shown).

[0273] The probes used were 4 femtomol of a oligonucleotide containing a tag at its 5'end and a CFTR sequence at its 3'end (SEQ ID Nr. 113 ; complement of nucleotides 389-418 of Genbank sequence M55116) and 10 nanogram of a digested single stranded M13 clone M234 containing 169 bp stuffer DNA between the sequence tag and the 42 nucleotides CFTR specific sequence (Complement of nucleotides 252-293 of Genbank sequence M55116). Clone M234 was prepared as described in example 1 by inserting a 169 bp PCR fragment of phage T7 DNA obtained with primers SEQ ID 108 + 109 in clone M214. In the clone obtained (M275), a double stranded synthetic DNA fragment (SEQ ID 110 + 111) was inserted. Single stranded DNA from the clone obtained (M245) was digested with EcoR5 and Bsm I in the presence of oligonucleotides SEQ ID 112 and SEQ ID 29 as described in example 1.

[0274] 0.5 ug human chromosomal DNA (Promega Corp.) was mixed with 4 femtomol probe SEQ ID 113 and 10 ng probe M245 and was diluted with water to 8.5 ul. The DNA was denatured by heating 5 minutes at 98 °C in a UNO 2 thermocycler with heated lid. To the DNA was added 1.5 ul of a salt solution (1500 mM KCl ; 300 mM Tris-HCl pH 8.5 ; 1 mM EDTA.). Annealing of the probes to the target DNA was for 16 hrs. at 60 °C in a thermocycler with heated lid. After decreasing the temperature to 37 °C, to the mixture was added 40 ul dilution-buffer (2 mM MgCl₂ ; 1 mM NAD⁺ ; 5 mM Tris-HCl pH 8.5 and 62,5 μM of each dNTP.) and 1.5 units sequenase. The mixture was incubated for 5 minutes at 37 °C. After increasing the temperature to 60 °C, 10 units Ligase-65 were added and incubation was for 15 minutes at 37 °C followed by 5 minutes enzyme inactivation at 95 °C. 10 ul of the mixture was used as a template for a PCR reaction in a 50 ul volume containing 2 units Taq polymerase ; 15 mM Tris-HCl pH 8.5 ; 50 mM KCl ; 1.5 mM MgCl₂ and 0.01 Triton X-100.

[0275] After heating the mixture to 65 °C, 10 pMol each of PCR primers SEQ ID NO. 55 and SEQ ID NO. 56 were added to provide a hot start.

[0276] PCR was performed in 200 ul tubes in a Biometra Uno 2 thermal cycler using the following conditions :

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- a) 2.5 minute denaturation at 95 C.
- b) 40 cycles consisting of 30 second denaturation at 95 C ; 30 second annealing at 60 C and 60 second elongation at 72 C.

5 [0277] Following the PCR reaction, 10 ul of this reaction was analysed on a 1.8 % agarose gel.

References :

- [0278] Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, van de T., Horres, M., Frijters, A., Pot, J., Peleman, J.,
10 Kuiper, M., and Zabeau, M. (1995) Nucleic Acid Research 23, 4407 - 4414 .
- [0279] Welsh, J. et al (1992) Nucleic Acid Research 20, 4965-4970.
- [0280] Zabeau, M., and Vos, P. (1992) European Patent Application 0534 858 A1.

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Annex to the applications documents - subsequently filed sequences listing

[0281]

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Claims

- 55 1. A method for detecting the presence of at least one single stranded target nucleic acid in a sample comprising providing said sample or sub-sample thereof, with a first nucleic acid probe complementary to a distinct part of said target nucleic acid and a second nucleic acid probe complementary to a second part of said target nucleic acid located essentially adjacent to said distinct part of said target nucleic acid, wherein said probes further comprise a tag which is essentially non-complementary to said target nucleic acid, the method further comprising

incubating said sample allowing hybridisation of complementary nucleic acids, connecting any essentially adjacent probes, amplifying any connected probe nucleic acid, wherein amplification is initiated by binding of a nucleic acid primer specific for said tag and detecting an amplicon, wherein at least one nucleic acid probe comprises enzymatic template directed polymerised nucleic acid prior to said connecting.

- 5 2. A method according to claim 1, wherein said connecting comprises ligation of the essentially adjacent probes.
- 10 3. A method according to claim 1 or claim 2, wherein said first and said second probe each comprise a different tag.
- 15 4. A method according to anyone of claims 1-3, further comprising detecting the presence of at least a second distinct target nucleic acid.
- 20 5. A method according to anyone of claims 1-5, further comprising providing said sample or sub-sample thereof, with at least a third nucleic acid probe complementary to a distinct part of said second target nucleic acid.
- 25 6. A method according to claim 5, further comprising providing said sample with at least a fourth nucleic acid probe complementary to target nucleic acid located essentially adjacent to said distinct part of said second target nucleic acid.
- 30 7. A method according to anyone of claims 4-6, wherein an amplicon is identifiable by mass, length and/or by the presence of a detection tag.
- 35 8. A method according to anyone of claims 1-7, further comprising providing said sample with at least one additional single stranded nucleic acid complementary to at least one interadjacent part of said target nucleic acid, whereby hybridisation of said additional nucleic acid to said interadjacent part allows the connecting of two adjacent probes.
- 40 9. A method according to anyone of claims 1-8, further comprising extending a 3' end of a hybridised probe prior to said connecting.
- 45 10. A method according to anyone of claims 4-9, wherein connected probes for at least two target nucleic acids can be amplified by the same primer pair.
- 50 11. A method according to anyone of claims 2-10, wherein said ligation is performed with a thermostable nucleic acid ligase capable of being inactivated within ten minutes above a temperature of approximately 90 °C.
- 55 12. A method according to anyone of claims 3-11, wherein at least one probe is generated by connecting at least two nucleic acids essentially in parallel with said connecting of essentially adjacent probes.
- 60 13. A method according to claim 12, wherein at least one of said at least two nucleic acid comprises enzymatic template directed polymerised nucleic acid prior to said connecting.
- 65 14. A method according to anyone of claims 1-13, wherein at least one probe is generated by digesting DNA, comprising said probe and/or a template therefor, with a restriction enzyme capable of cutting outside the enzyme recognition site sequence on said DNA.
- 70 15. A method according to anyone of claims 4-14, further comprising providing said sample with a competitor nucleic acid comprising a nucleic acid sequence capable of competing with at least one probe for hybridisation to a target nucleic acid.
- 75 16. A method according to anyone of claim 1-15, wherein said sample is further provided with a known amount of one or more connected probes, prior to said amplification.
- 80 17. A method according to anyone of claims 1-16, further comprising quantitation of the (relative) abundance of target nucleic acid in said sample or sub-sample.
- 85 18. Use of a method according to anyone of claims 1-17, for detecting a nucleotide polymorphism.
- 90 19. Use according to claim 18, for detecting a single nucleotide polymorphism.

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20. Use according to claim 18 or claim 19, for the detection of multiple single stranded target nucleic acids.
21. A use according to claim 20, wherein said multiple single stranded target nucleic acids are detected through the detection of multiple amplicons.
- 5 22. A use according to claim 21, wherein at least two of said multiple amplicons can be discriminated on the basis of a difference in size of said at least two amplicons.
- 10 23. A use according to anyone of claims 18-21, for determining the (relative) abundance of multiple single stranded target nucleic acids in said sample or sub-sample.
24. A use according to anyone of claims 1-17 for the amplification of a breakpoint region in rearranged nucleic acid.
- 15 25. A nucleic acid probe for use in a method according to anyone of claims 1-17.
26. A mixture of nucleic acids comprising two or more probes according to claim 25.
27. A kit for performing a method according to anyone of claims 1-17, comprising a nucleic acid probe according to claim 25 or a mixture of probes according to claim 26.
- 20 28. A method for ligating at least two nucleic acid to each other comprising incubating a sample comprising said nucleic acids with a thermostable nucleic acid ligation enzyme under suitable conditions, wherein said ligation enzyme is capable of being essentially inactivated by incubating said sample for 10 minutes at a temperature of approximately 90 °C.
- 25

30

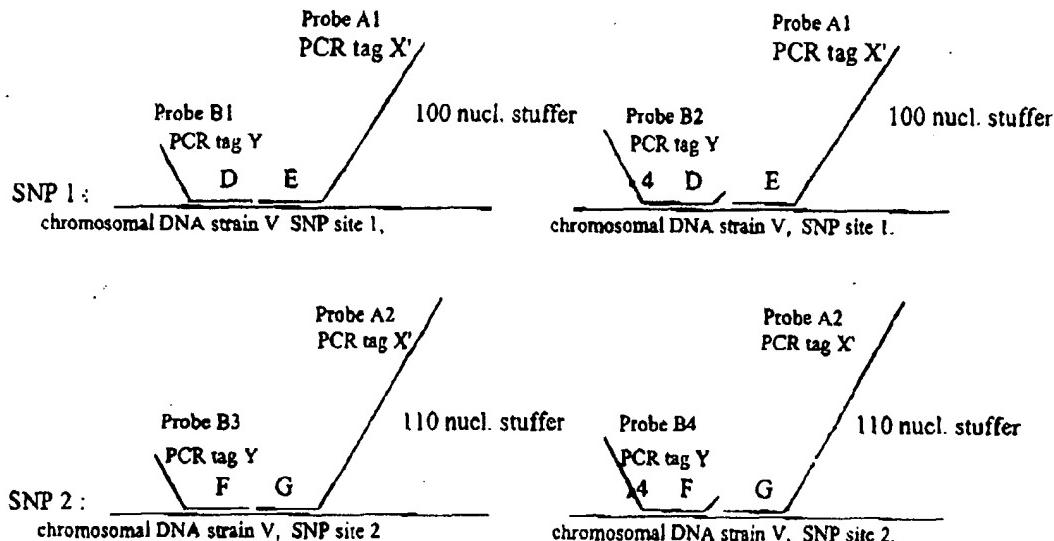
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40

45

50

55

Figure 1, part 2.**Annealing of probes to denatured chromosomal DNA of strain V, and Ligation of probes :****Ligation products with DNA of strains V and W used as ligation template :**

Strain	SNP	Probes	Relative amount	Length of amplification product with primers X and Y
V	1	: A1 + B1	Large	Y + D + E + 100 + X = 180 bp.
	1	: A1 + B2	Small	Y + 4 + D + E + 100 + X = 184 bp.
	2	: A2 + B3	Large	Y + F + G + 110 + X = 190 bp.
	2	: A2 + B4	Small	Y + 4 + F + G + 110 + X = 194 bp.

Strain	SNP	Probes	Relative amount	Length of amplification product with primers X and Y
W	1	: A1 + B1	Small	Y + D + E + 100 + X = 180 bp.
	1	: A1 + B2	Large	Y + 4 + D + E + 100 + X = 184 bp.
	2	: A2 + B3	Small	Y + F + G + 110 + X = 190 bp.
	2	: A2 + B4	Large	Y + 4 + F + G + 110 + X = 194 bp.

Sequencing type gel : DNA from strain V as ligation template :



DNA from strain W as ligation template :

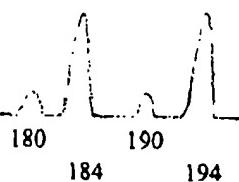


FIGURE 2 : Graphic Outline of the MLDA Invention.

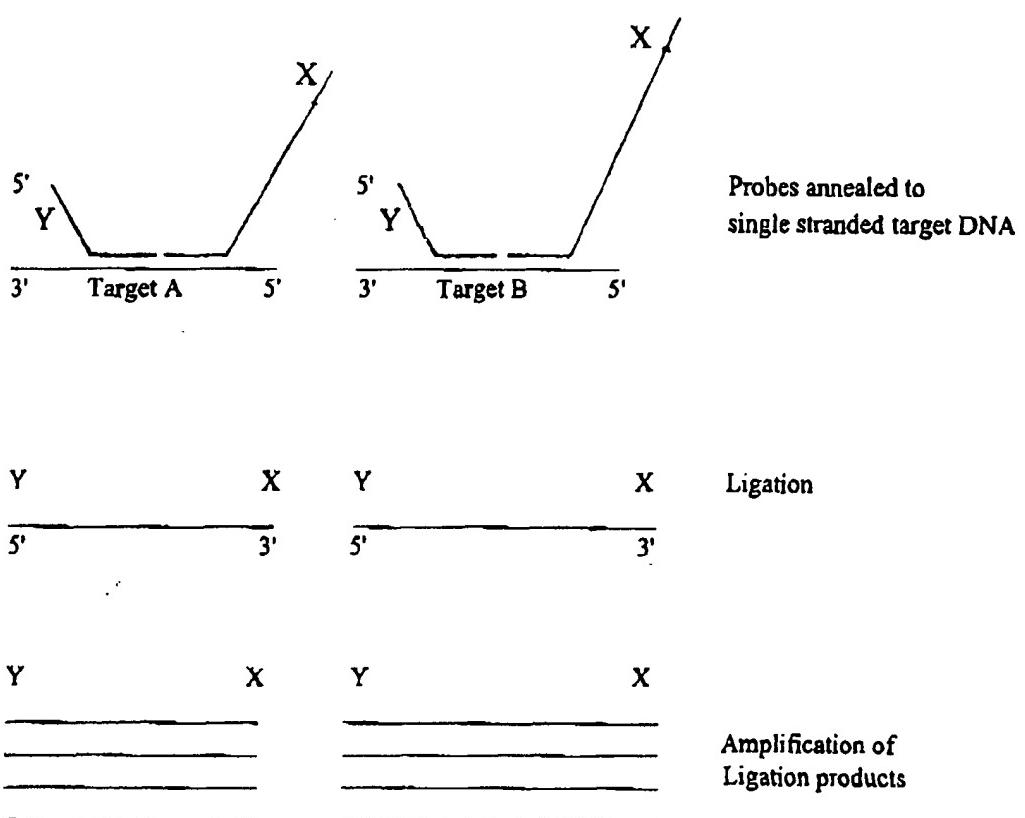


FIGURE 3 : Graphic outline of the MLDA invention for mutation detection.

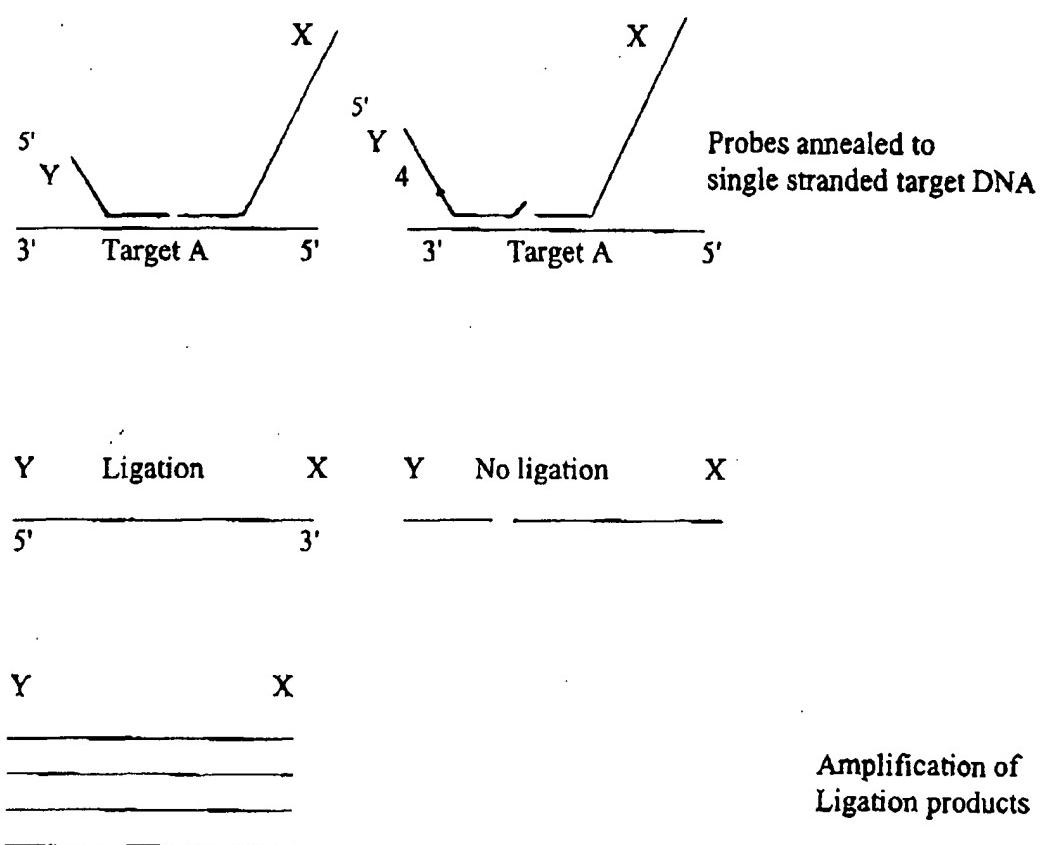


FIGURE 4 : Graphic outline of a M13 clone used to prepare long Probes.

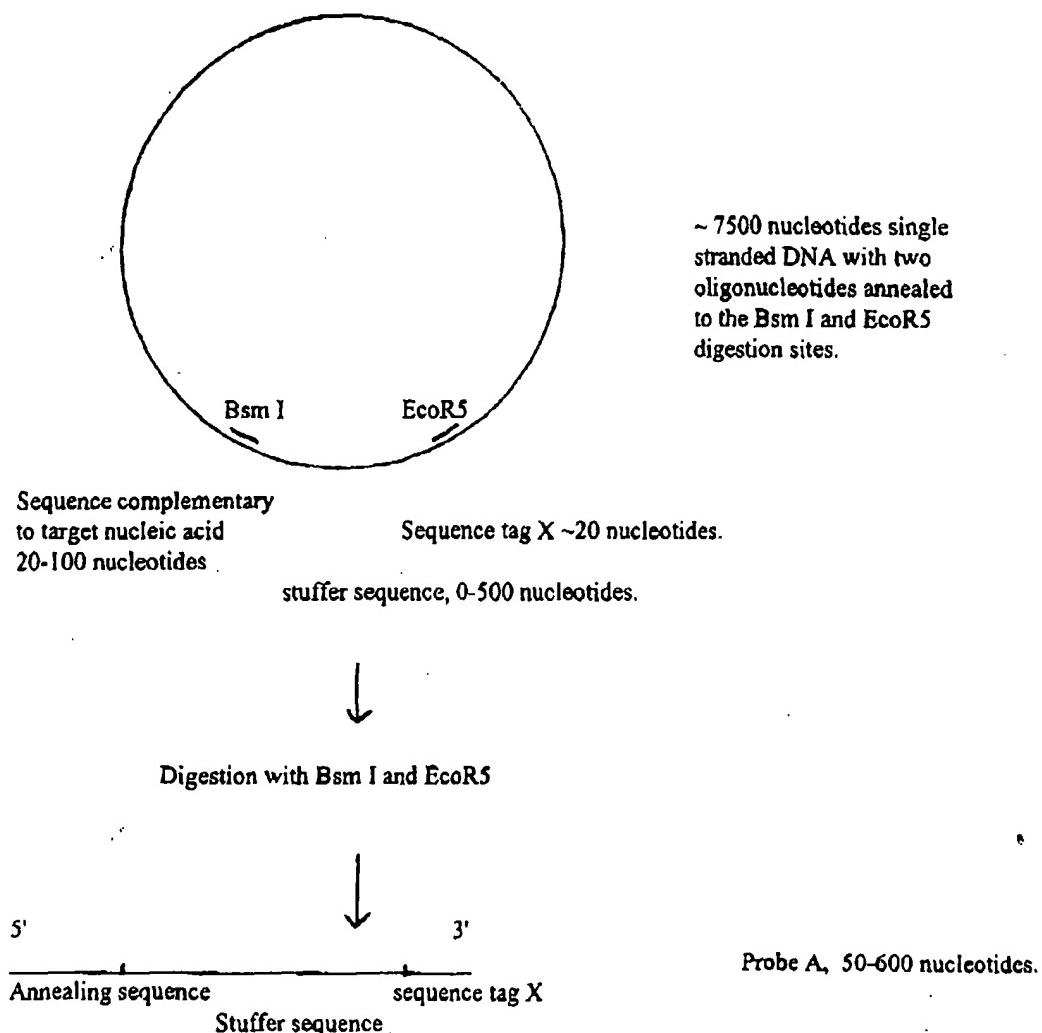


FIGURE 5 : Simplified way of performing the MLDA invention.

Probes provided in the MLDA assay:

Target sequence	A probe	wild type specific Probe type B	mutant specific Probe type B	Length of amplification product
Control sequence 1	Yes	Yes	-	150 bp.
Control sequence 2	Yes	Yes	-	350 bp.
Mutation site 1 (abundant)	Yes	No	Yes	200 bp.
Mutation site 2 (rare)	Yes	No	Yes	250 bp
Mutation site 3 (rare)	Yes	No	Yes	250 bp.
Mutation sites 4-100	Yes	No	Yes	250 bp.

Except for the control target sequences, no type B probes specific for wildtype sequences are used.

Results obtained on agarose gel electrophoresis :

Lane	1	2	3	4	5	6	7	8	9	10	11	12
Control band	-	-	-	-	-	-	-	-	-	-	-	-
Rare mutation								-				
Abundant mutation					-							
Control band	-	-	-	-	-	-	-	-	-	-	-	-

Conclusion: Sample 5 contains the abundant mutation.
 Sample 8 contains one of the 99 rare mutations
 MLDA assay on sample 11 failed
 Other samples do not contain any of the 100 mutations tested.

FIGURE 6 : Use of the MLDA invention for the detection of mRNA's.

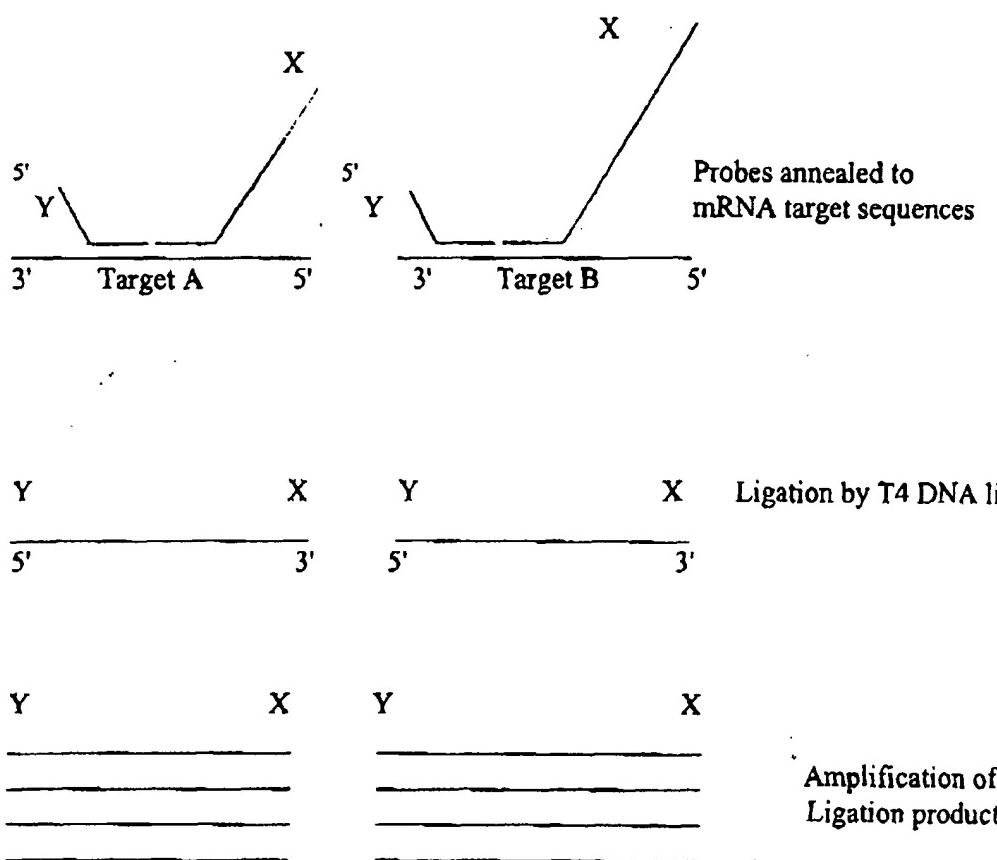


FIGURE 7 : Detection of cDNA target sequences.

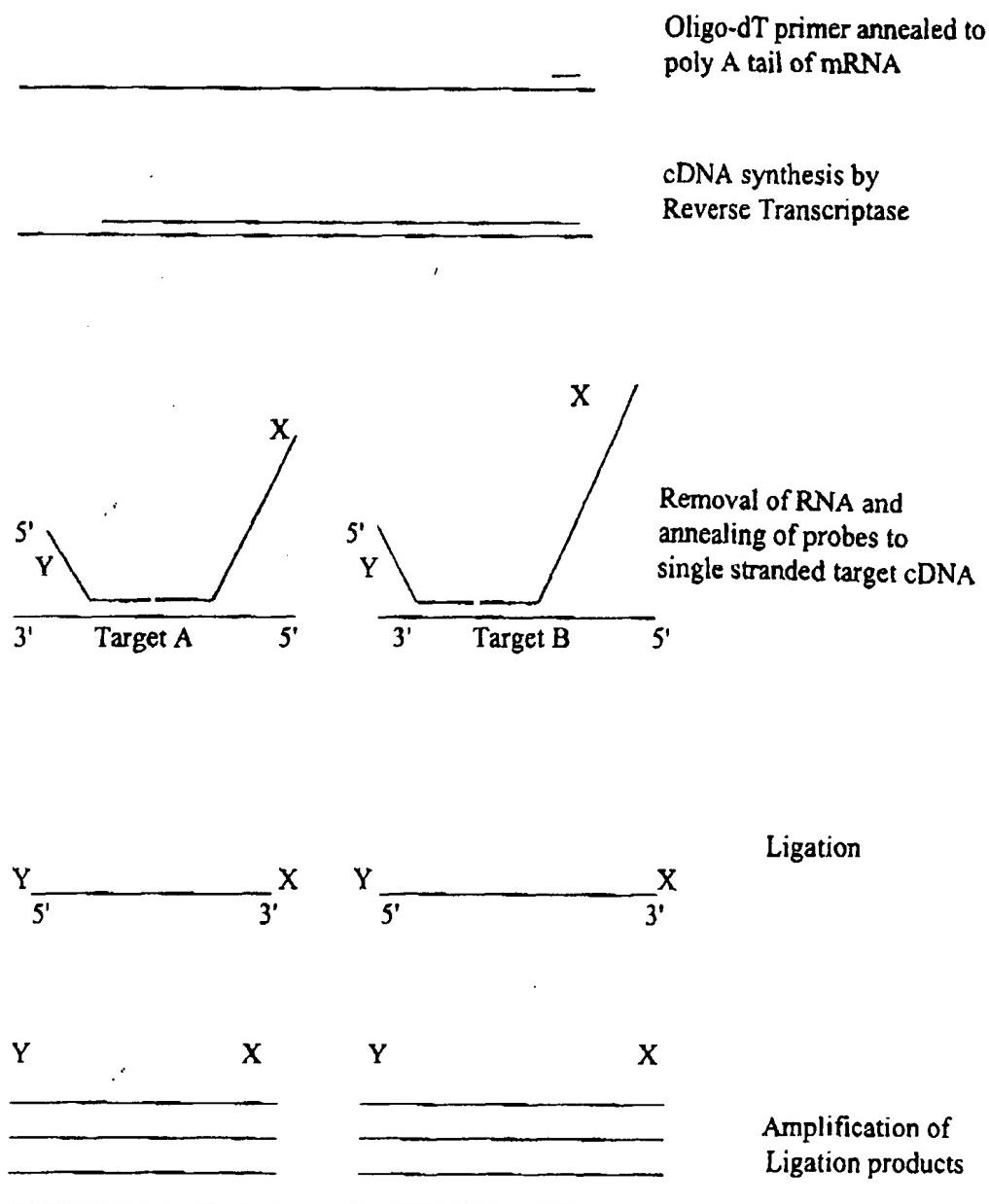


FIGURE 8 : Detection of cDNA target sequences using gene specific reverse transcription primers.

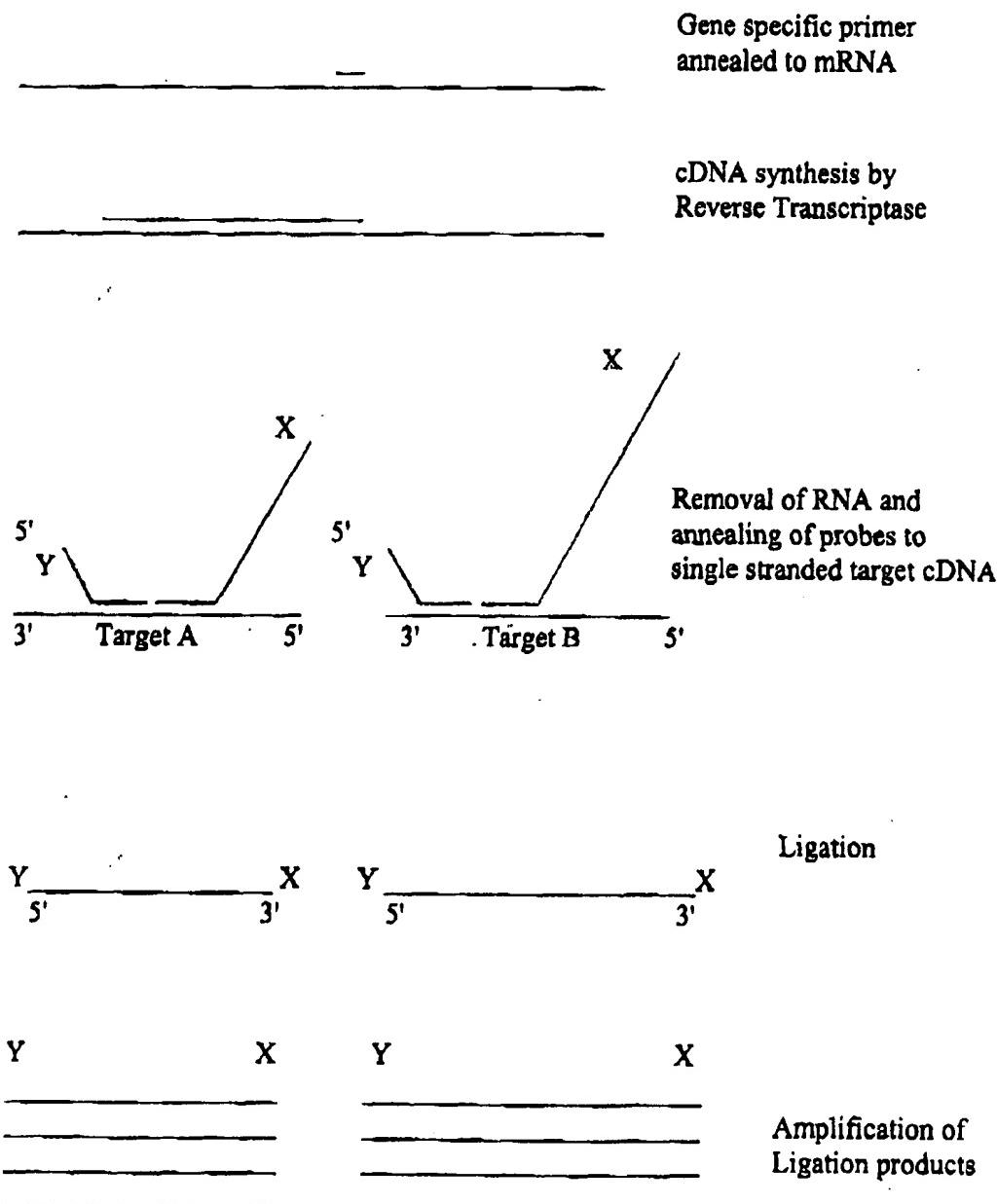


FIGURE 9 : Detection of cDNA target sequences using tagged gene specific reverse transcription primers.

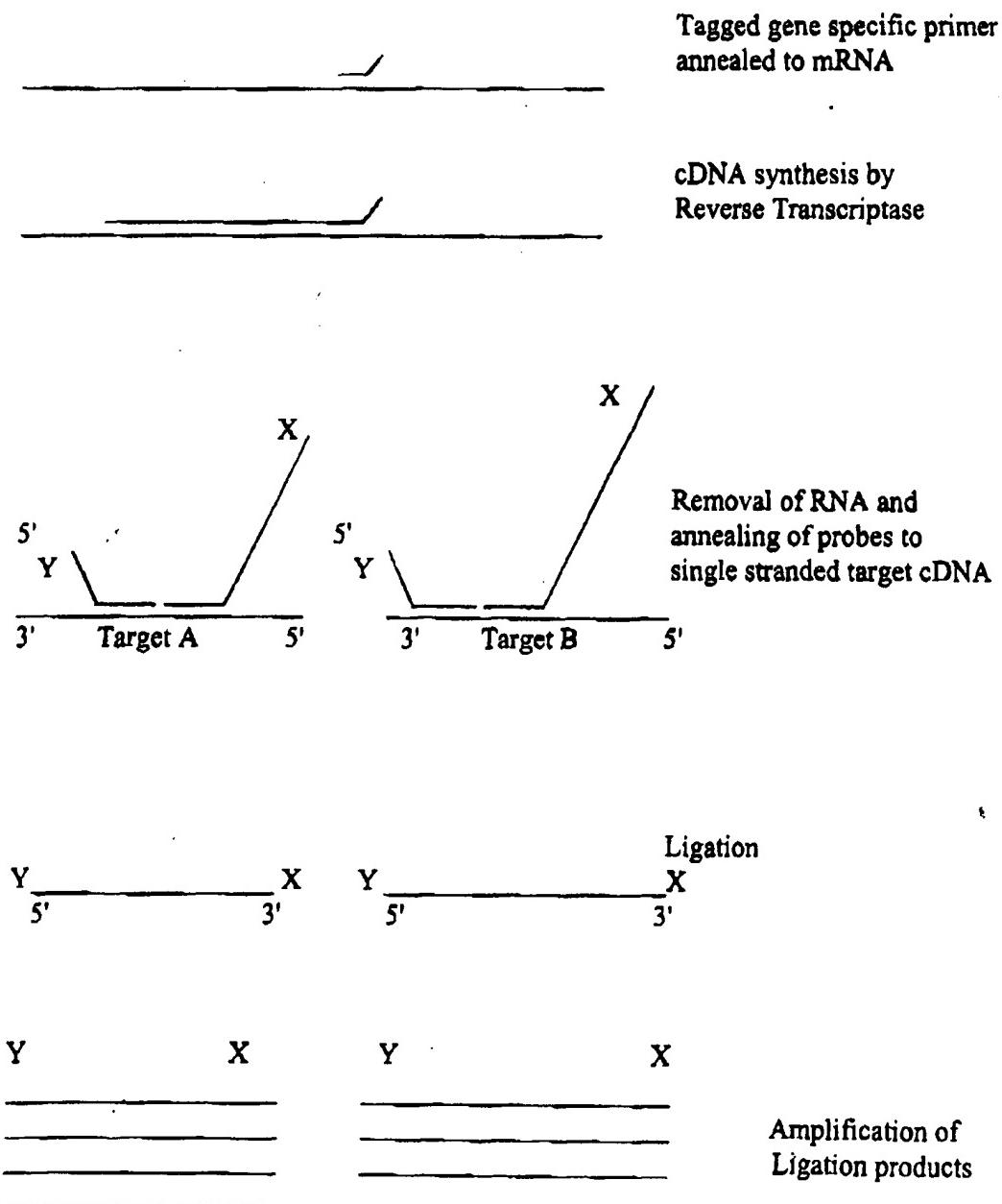


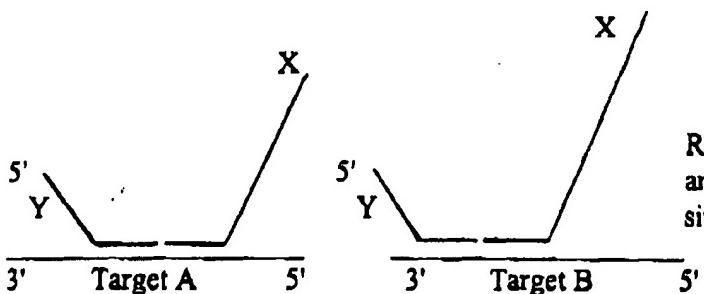
FIGURE 10 : Detection of cDNA target sequences using tagged gene specific reverse transcription primers.

Annealing of sequence tagged reverse transcription primer + biotinylated complementary oligonucleotide to target RNA in whole cell lysates

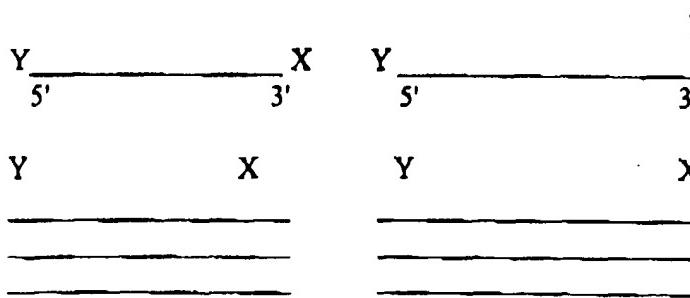
Purification of biotin containing complexes with immobilised streptavidine.

Elution at a temperature at which the RT primer-RNA complex is not dissociated

cDNA synthesis by Reverse Transcriptase



Removal of RNA and annealing of probes to single stranded target cDNA



Amplification of Ligation products

FIGURE 11 : The use of reverse transcriptase primers that are part of one of the probes.

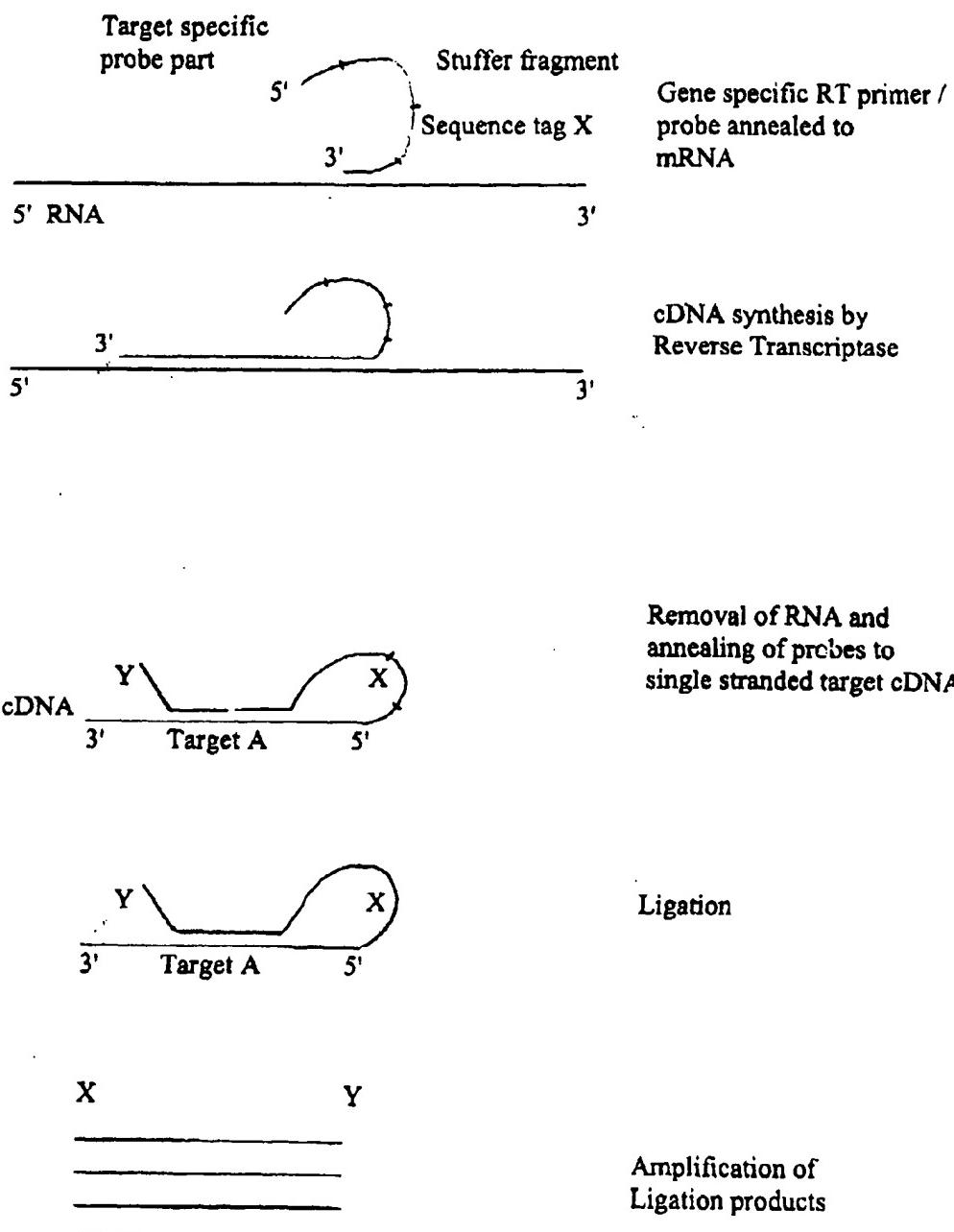


FIGURE 12 : The use of the MLDA invention without the use of target specific clones.

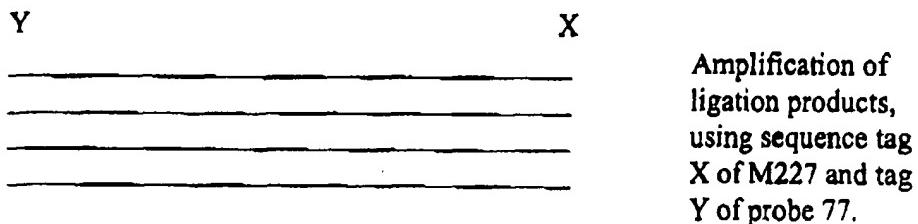
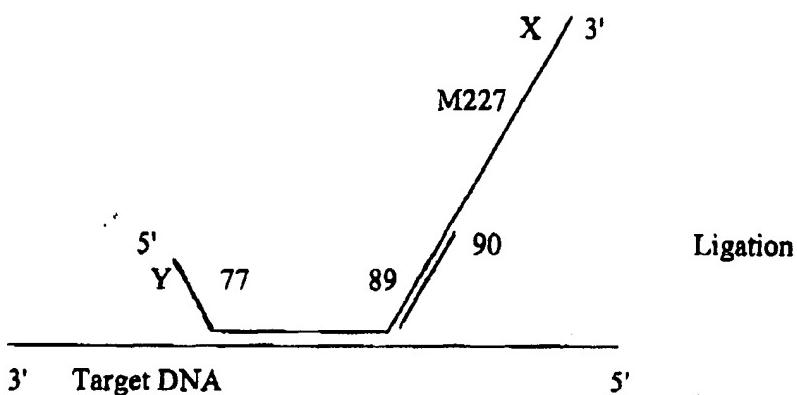
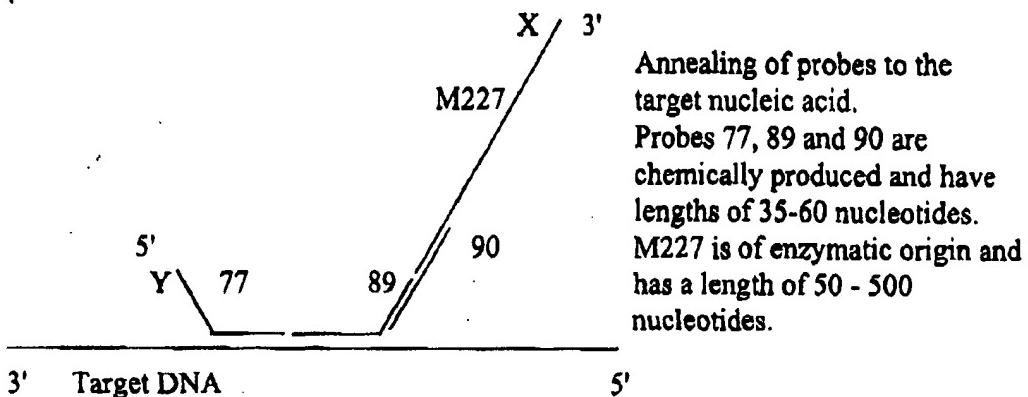


FIGURE 13 : Alternative way of performing the MLDA invention.

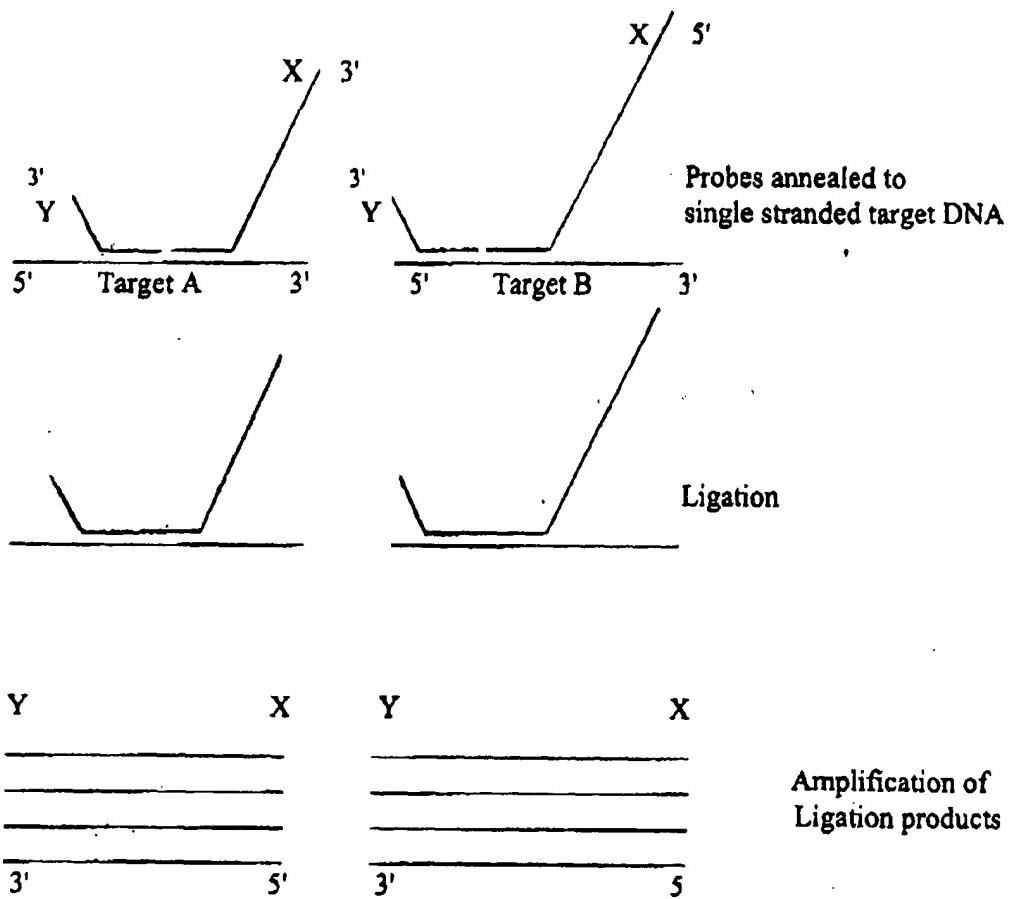


FIGURE 14 : The use of "viagra" oligonucleotides to reduce internal secondary structures of the probes.

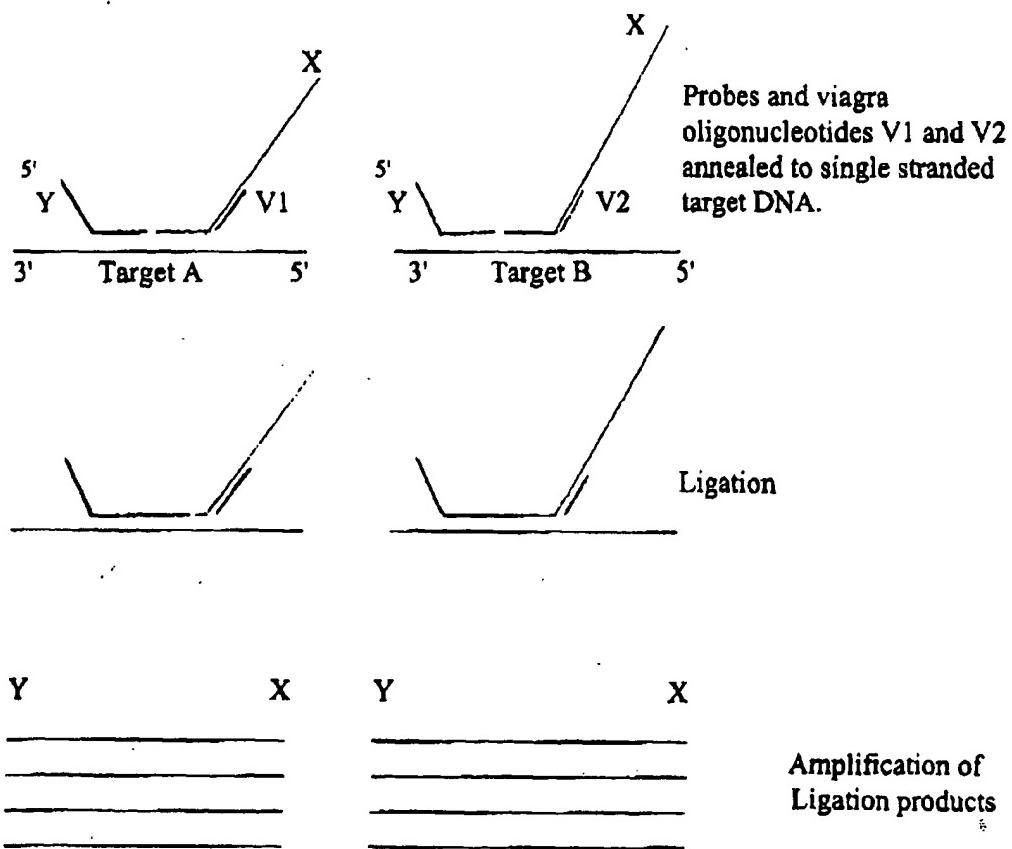


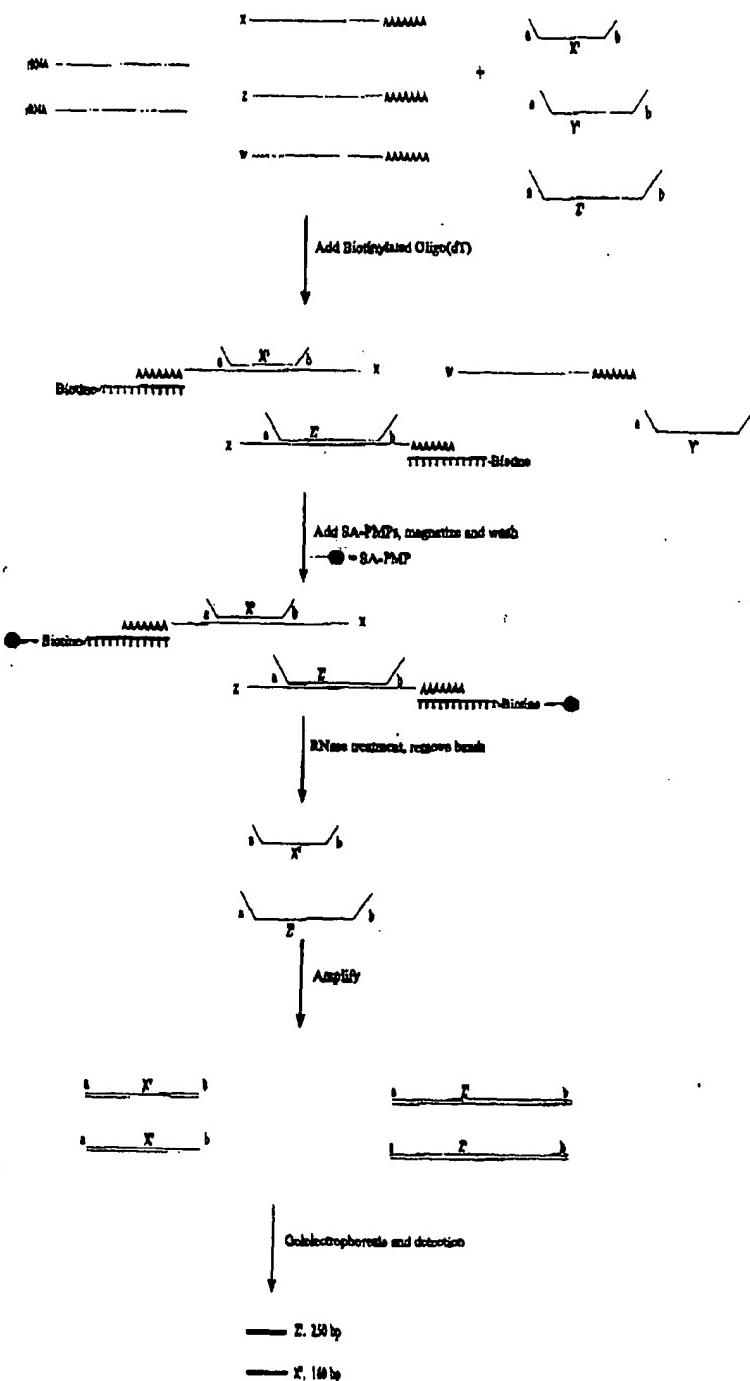
FIGURE 15 : mRNA detection and relative quantification with complete probes.

FIGURE 16.

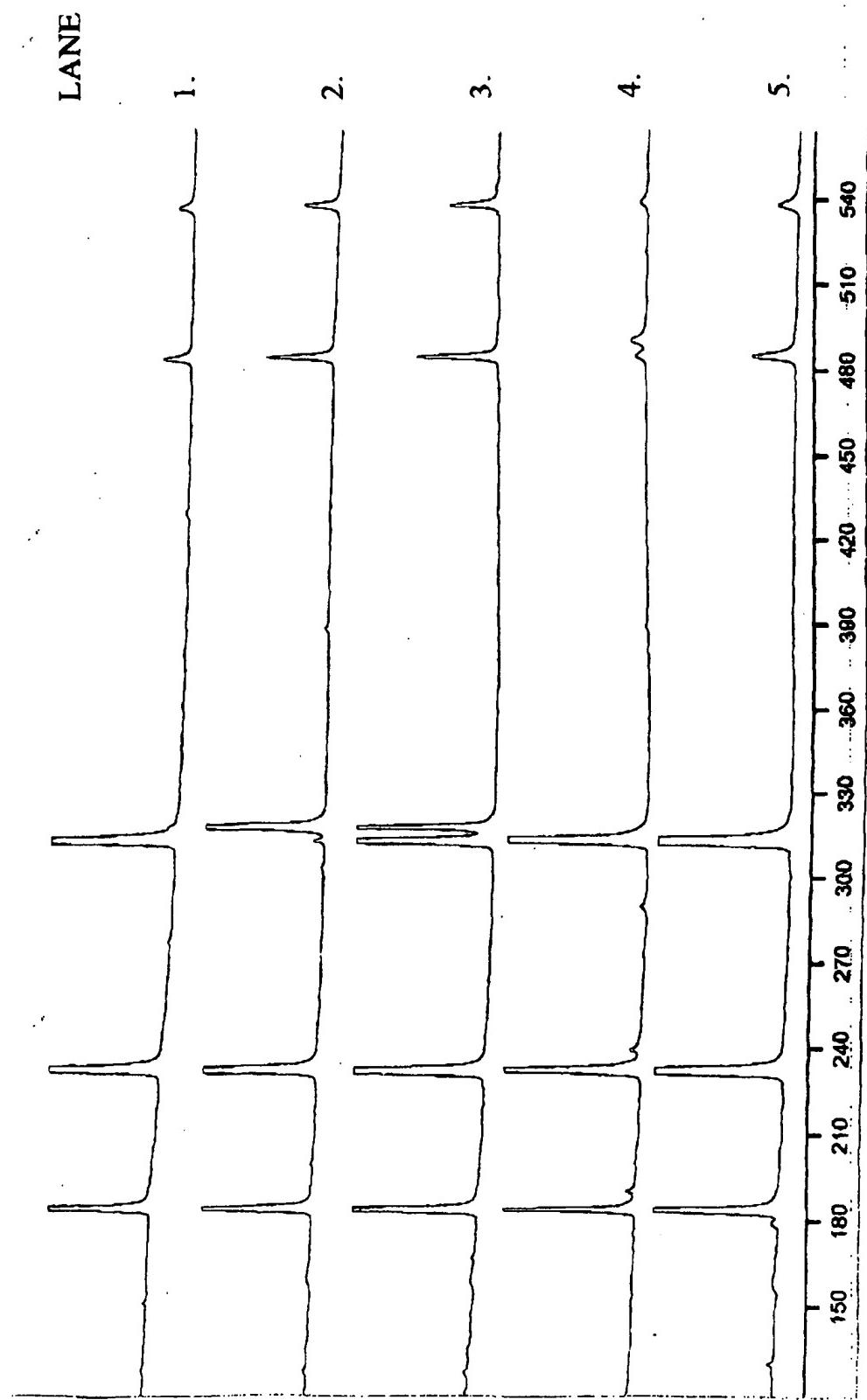


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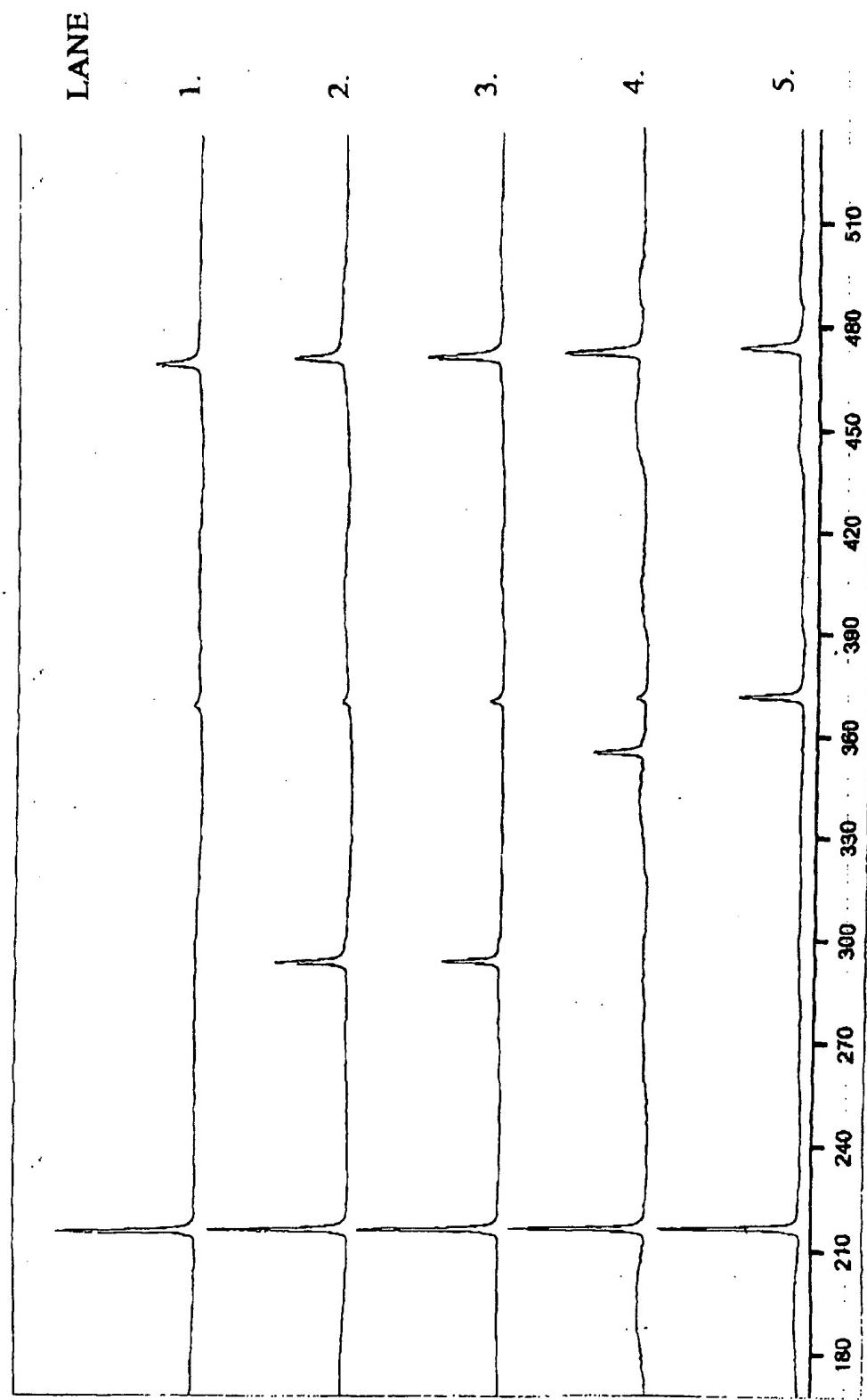


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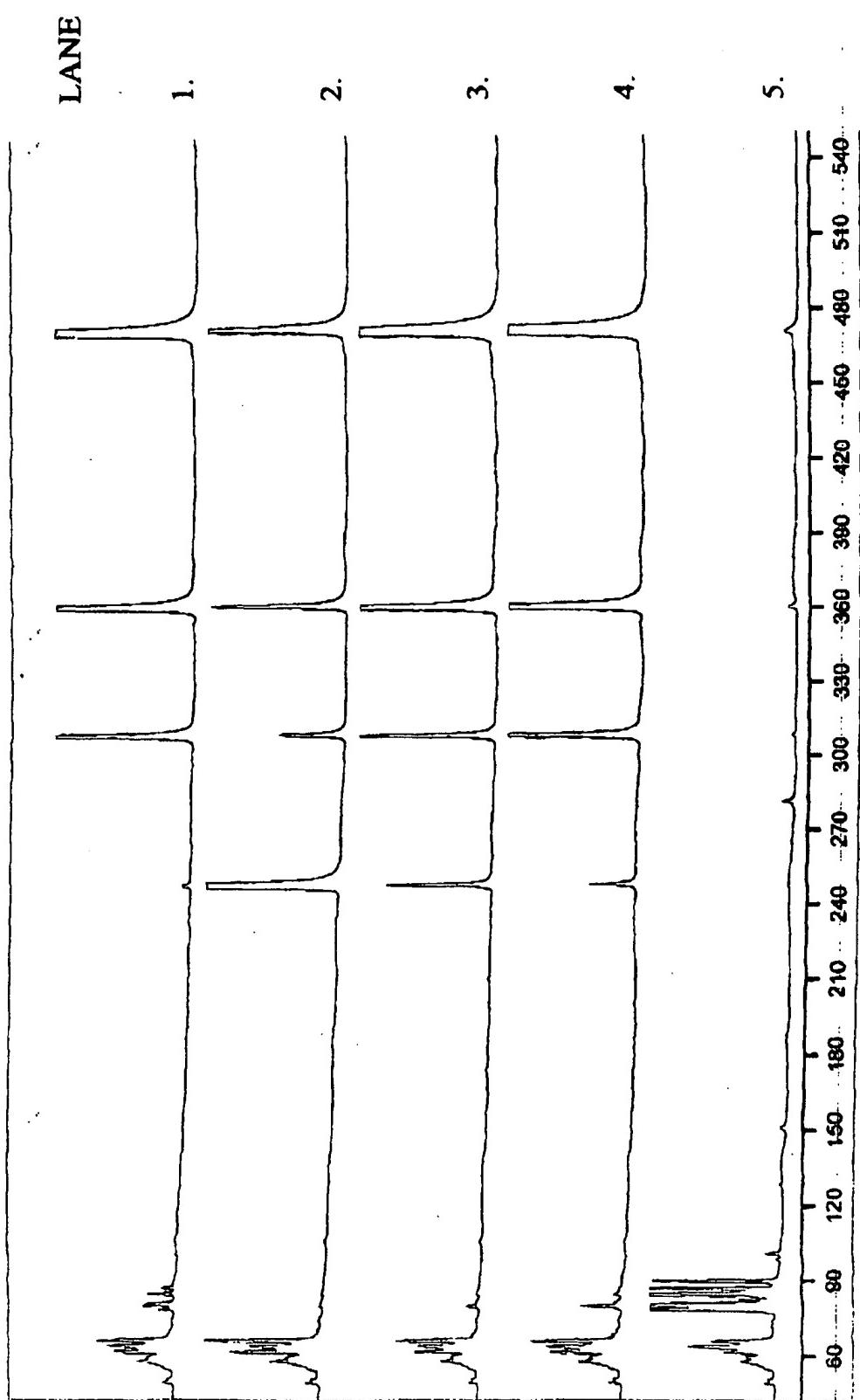


FIGURE 19.

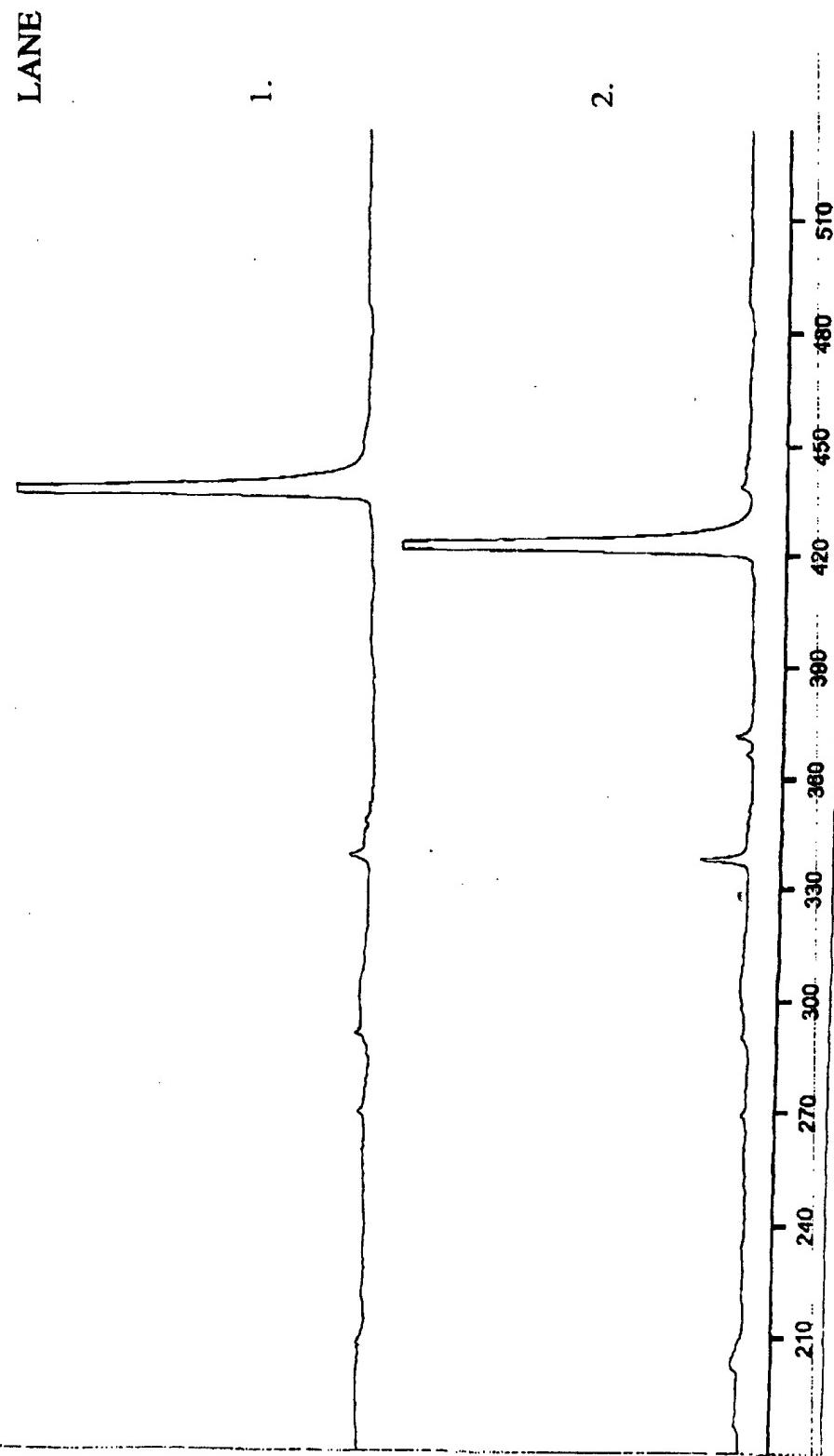


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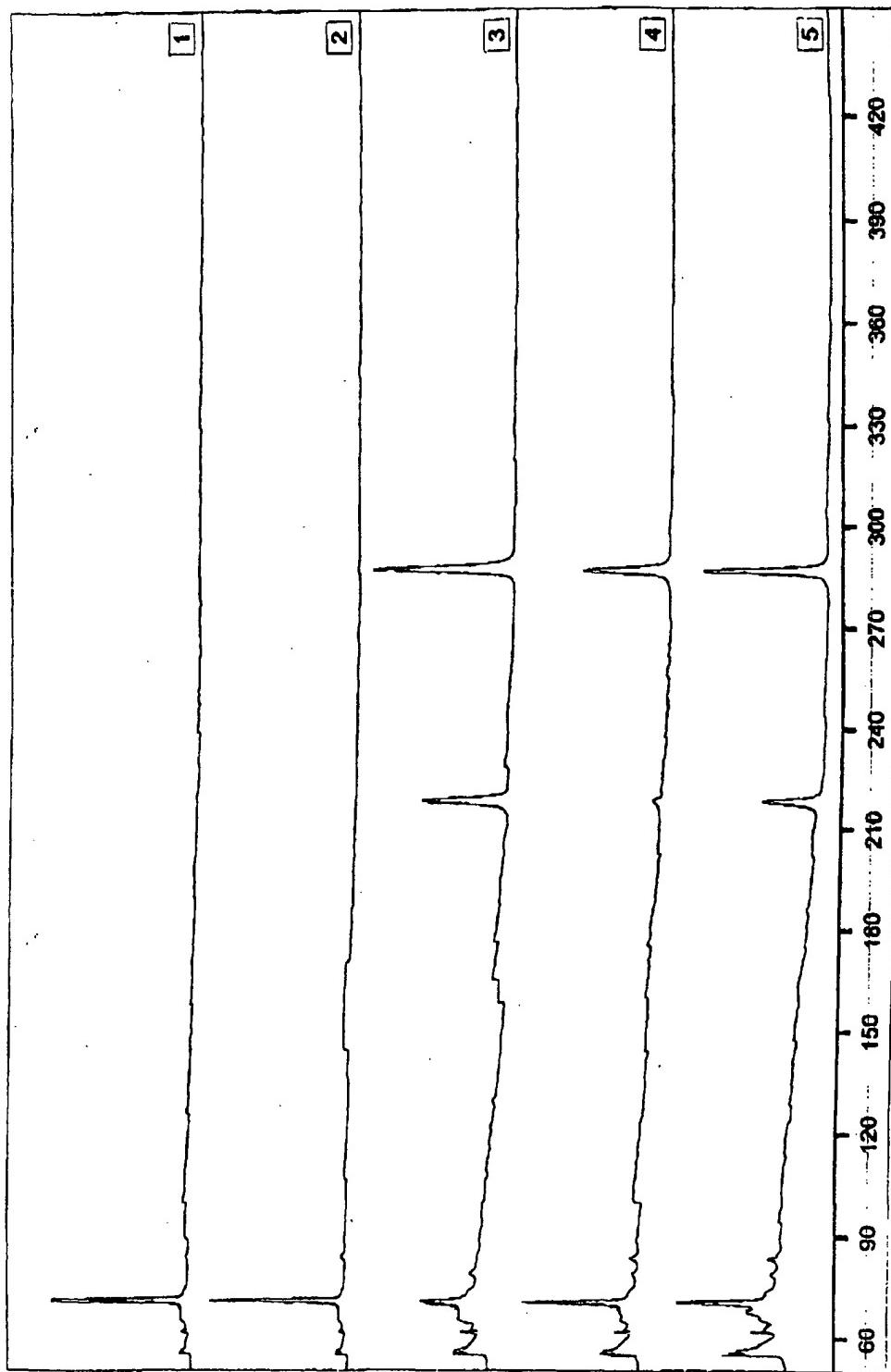


FIGURE 21 : Alternative way of performing the MLDA invention.

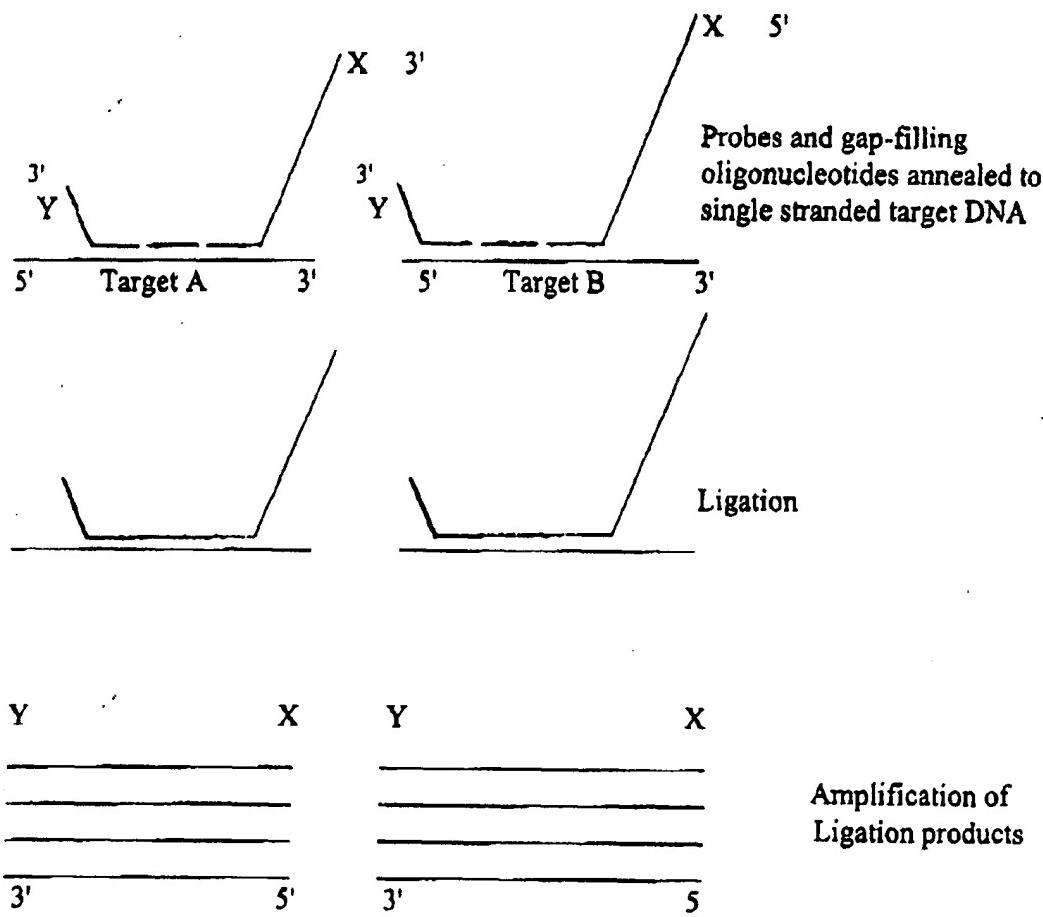


FIGURE 22 : Alternative way of performing the MLDA invention.

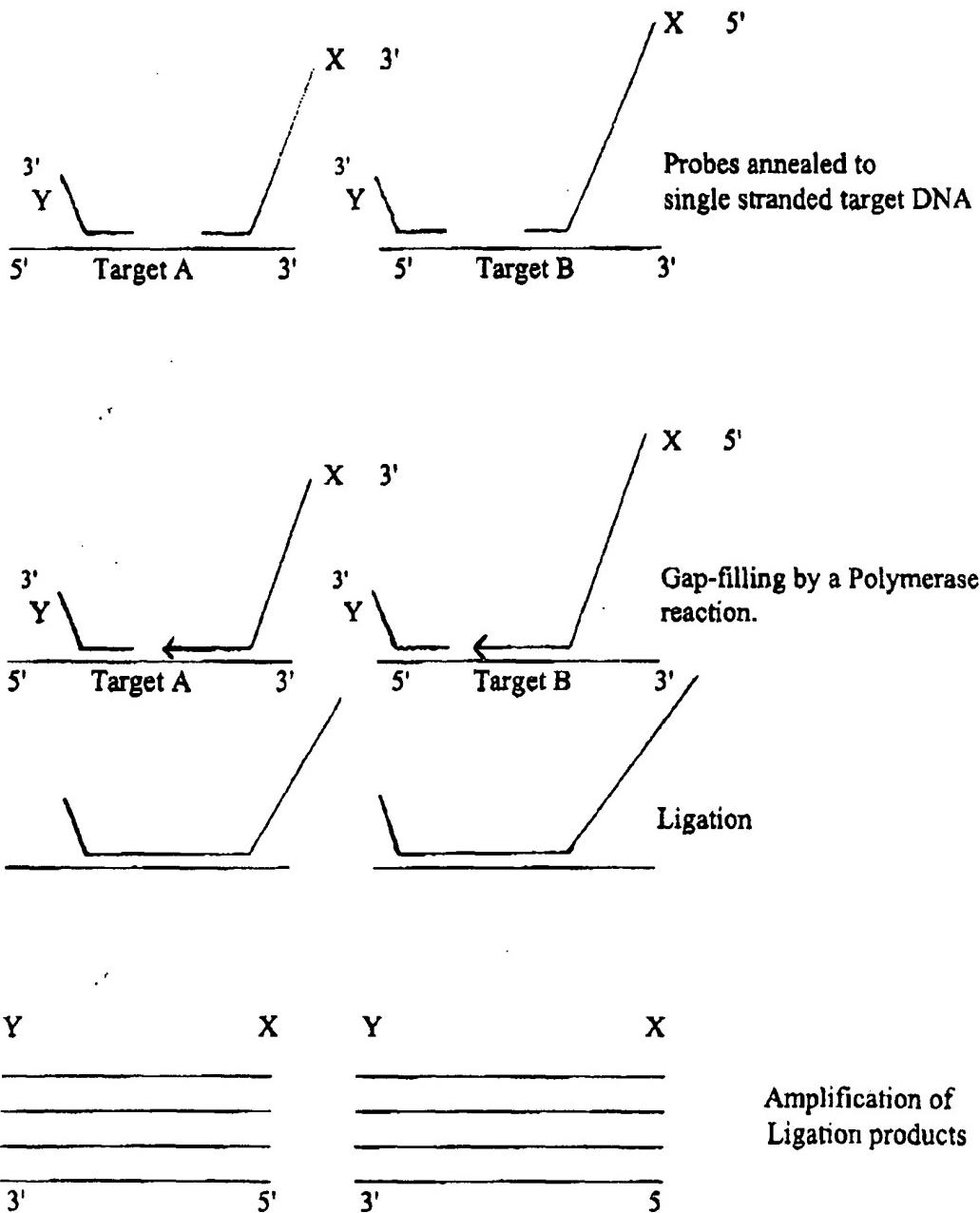


FIGURE 23 : The use of the MLDA invention for the determination of the break-point site in chromosomal rearrangements.

Wildtype DNA + Probes:

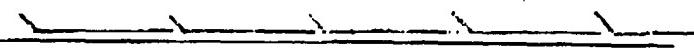


ABL:

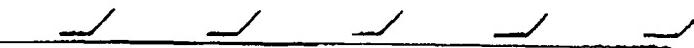


Elongation of Probes:

BCR:



ABL:



No ligation events possible ; No exponential amplification of ligated probes.

Rearranged DNA + Probes:

BCR



ABL

Elongation of Probes:

BCR



ABL

Ligation of probes:

BCR



ABL

Amplification of the fragment spanning the breakpoint.

Sequence determination of the amplified fragment to confirm that it contains both BCR and ABL sequences, and to determine the exact breakpoint.

Design of (nested) PCR primers specific for the rearranged DNA.

Figure 24

SEQ ID NO:1: CGGCGTCGAG ACTAGACCGG CTGGGTGGC ACAGCCTG

SEQ ID NO:2: CCCCGGCCAG CAAGATCCGA CAGGCGGAGC AGCATGAG

SEQ ID NO:3: CGGCGTCGAGACTAGACCGGGAAAGGCGACAGTGCCTAAG

SEQ ID NO:4: CCCCGGCCAGCAAGATCCTAGGTCTTCAGGAGCTGATCAACA

SEQ ID NO:5: FITC-GGCGTCGAGACTAGACCG

SEQ ID NO:6: GACGCGCCAGCAAGATCC

SEQ ID NO:7: biotin-

TT

SEQ. ID NO. 8 : AATTTCGCGATATCCCATGGCTTAAGAGTCGACTCGCGATATC

SEQ. ID NO. 9 : AGCTGATATCGCGAGTCGACTCTTAAGCCATGGGATATCGCGA

SEQ. ID NO. 10 : CATGGCGTCGAGACTAGACCGAATTGAGCGCGCAAAGCTT
GGATCTTGCTGGCGCGT

SEQ. ID NO. 11 : CGACGCGCCAGCAAGATCCAAGCTTGCGCGCTCGAATTGG-
TCTAGTCTCGACGC

SEQ ID NO. 12 : CCTGTAGCGTTCCACAGACAACCCCTC

SEQ ID NO. 13 : GGAACGCTACAGGC GTTAGTTGTACTG

SEQ. ID NO. 14 : CATGGCAGTTCGAACTTGAATGCCTTAGAGTACTCAT
CACCGGTTCTGG

SEQ. ID NO. 15 : AATTCCAGAACCGGTGATGAGTACTCTAAGGCATT
CAAGTTCGAACTGC

SEQ. ID NO. 16 : CCGGTGCATGCTTAGCGCCACTCTA-
GATTGGATCTTGCTGGCGCGT

SEQ. ID NO. 17 : CGACGCGCCAGCAAGATCCAATCTAGAGTGCGCGCTAAG-
CATGCA

SEQ ID NO. 18 : GAATTCGCG ATATCCATG GCAGTTGAA CTTGAATGCC
TTAGAGTACT CATCACCGGT GCATGCTTAG CGCGCACTCT AGATTGGATC
TTGCTGGCGC GTGCACTCGC GATATCAGCT T

SEQ. ID NO. 19 : CACCACGCATGCTGCCATAGTCGCCTTCA

SEQ. ID NO. 20 : CACAACCTCTAGACTGATAATCAACGTCCCTCAGG

SEQ. ID NO. 21 : CACCACGCATGCTGGCGTGGTCAACTC

SEQ. ID NO. 22 : CAACCTCTAGACTCCCTCAAGTTAACACCG

SEQ. ID NO. 23 : CACCACGCATGCCGTAGCATGACTGGTGG

SEQ. ID NO. 24 : CACAACCTCTAGAAACGTCAGCCGTAGGA

SEQ. ID NO. 25 : CACCACGCATGCCGTGGATGACCGCGATG

SEQ. ID NO. 26 : CACAACCTCTAGAACGGTCTGCTTGTGTT

SEQ. ID NO. 27 : CCCGCTTATTGTTAACCTACTGCGGCATAGAGTCT

SEQ. ID NO. 28 : CTAGAGACTCTATGCCGAGTAGGTTCAACAATAAGCGGGCATG

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SEQ ID NO. 109 : CACCTCTAGACTCATGGATGTCAGAACGCTG

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SEQ ID NO. 113 :
GGGTTCCCTAAGGGTTGGAGCTTGCTAGACCAATAATTAGTTATTCAACC



European Patent
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EUROPEAN SEARCH REPORT

Application Number
EP 00 20 0506

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim							
X	WO 96 15271 A (ABBOTT LAB) 23 May 1996 (1996-05-23) * the whole document *	1-28	C12Q1/68						
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<p>The present search report has been drawn up for all claims</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">Place of search</td> <td style="width: 33%;">Date of completion of the search</td> <td style="width: 34%;">Examiner</td> </tr> <tr> <td>THE HAGUE</td> <td>18 August 2000</td> <td>Hagenmaier, S</td> </tr> </table> <p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons B : member of the same patent family, corresponding document</p>				Place of search	Date of completion of the search	Examiner	THE HAGUE	18 August 2000	Hagenmaier, S
Place of search	Date of completion of the search	Examiner							
THE HAGUE	18 August 2000	Hagenmaier, S							

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EP 00 20 0506

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